Sorghum bicolor defence responses to the pathogen *Burkholderia andropogonis*: An LC-MS based metabolomic analysis

by

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"Now unto him that is able to do exceeding abundantly above all that we ask or think, according to the power that worketh in us"

~Ephesians 3:20

Table of Contents

Dedication

The work in this dissertation is dedicated to parents,

Judith Mareya (nee Matete) – my beloved mother and Barnabas Nyangadzai Mareya – my beloved father, I am truly grateful for all the immeasurable sacrifices, unwavering prayers, care, love and boundless support throughout this journey – being my pillar of strength and source of inspiration.

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"No one who achieves success does so without acknowledging the help of others. The wise and confident acknowledge this help with gratitude"

–Alfred North Whitehead

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List of abbreviations

1,2-SnGlc	1,2-Bis-*O*-sinapoyl-beta-D-glucoside
3-FQA	3-Feruloylquinic acid
4CL	Coumarate 4-ligase
4-*p*CoAg	4-Coumaroylagmatine
4-*p*CoQA	4-Coumaroylqunic acid
7GT	Flavanone-7-*O*-glucosyltransferase
ABA	Abscisic acid
ANS	Anthocyanidin synthase
BRs	Brassinosteriods
BT	Bitter/NS 5511
C4H	Cinnamate 4-hydroxylase
CCA	Canonical correlation analysis
CCL	Coumaroyl-CoA ligase
CE-MS	Capillary electrophoresis-mass spectrometry
CGT	*C*-glycosyl transferase
CHI	Chalcone isomerase
CHS	Chalcone synthase
CID	Chemical identifiers
CKs	Cytokinins
Cm1,2RhaT	1,2-Rhamnosyltransferase
cv	Cultivar
CV-ANOVA	Cross validated analysis of variance
DAMPs	Damage/danger-associated molecular patterns
DFR	Dihydroflavonol reductase
DH	Dehydrogenase
DIMS	Direct infusion mass spectrometry
DModX	Distance to the model in space X
DNA	Deoxyribonucleic acid
EF-Tu	Elongation factor Tu
ESI	Electrospray ionisation
ET	Ethylene
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility

F2HI	Flavanone-2-hydrohylase
F3′ H	Flavonoid 3′ hydroxylase
FA	Ferulic acid
FGRT	Flavonol-3-*O*-glucoside L-rhamnosyltransferase
FGT	Flavonoid 3-*O*-glucosyltransferase
FHS	Flavonoid 3', 5'-hydroxylase
FLS	Flavonol synthase
FLS2	Flagellin-sensitive 2
FND	Flavanone 3-dioxygenase
FNR	Flavanone 4-reductase
FNS	Flavone synthase
FRG	Flavanone 7-*O*-glucoside-2"-*O*-β-L-rhamnosyltransferase
FT/IR	Fourier transform-infrared
GAs	Gibberellins
GC	Gas chromatography
GH	Grade - High condensed tannin (sorghum seed)
GM	Grade – Malting (sorghum seed)
HAMPs	Herbivore-associated molecular patterns
HCA	Hierarchical cluster analysis
HGD	2-Hydroxynaringenin-6-*C*-glucoside dehydratase
HR	Hypersensitive response
HRMS	High-resolution, accurate-mass MS
IAA	Indole-3 acetic acid/ auxin
iFit	Isotopic fit
IR	Induced resistance
ISR	Induced systemic resistance
IT-Q-TOF-MS	Ion trap-quadrupole-time-of-flight-MS
JA	Jasmonic acid
JA-Ile	Jasmonoyl-L-isoleucine
LAR	Local acquired resistance
LC-MS	Liquid chromatography coupled to mass spectrometry
LDA	Linear discriminant analysis
LGR	Luteolin 2-*O*-β-L-rhamnosyltransferase;
LORE	Lipo-oligosaccharide-specific Reduced Elicitation (mutant)
LPS	Lipopolysaccharides
$LPS_{B.\ andr.}$	*Burkholderia andropogonis* LPS
$LPS_{B.\ cep.}$	*Burkholderia cepacia* LPS

LRR-RKs	Leucine-rich repeat receptor-like kinases
M/PAMPs	Microbe/pathogen-associated molecular patterns
M/PTI	M/PAMP-triggered immunity
m/z	Mass-to-charge
MAPK	Mitogen-activated protein kinase
Mbp	Mega base pairs
MeJA	Methyl jasmonate
MEP	Methylerythritol phosphate pathway
MeSA	Methyl salicylate
MSI	Metabolomics Standard Initiative
MT	Methyltransferase
MVDA	Multivariate data analysis
NAMPs	Nematode-associated molecular patterns
NB-LRR	Nucleotide-binding –leucine rich repeats
NMR	Nuclear magnetic resonance
NO	Nitric oxide
OD	Optical density
OGT	*O*-glycosyltransferase
OM	Outer membrane
OPLS-DA	Orthogonal partial least squares discriminant analysis
OPS	O-polysaccharide/O-antigen
PAGE	Polyacrylamide gel electrophoresis
PAL	Phenylalanine ammonia-lyase
PCA	Principal component(s) analysis
PCD	Programmed cell death
***p*CoA**	*p*-Coumaric acid
***p*CoGlc**	1-O-Coumaroyl-beta-D-glucose
PCs	Principal components
PDA	Photodiode array
PGPF	Plant growth-promoting fungi
PGPR	Plant growth-promoting rhizobacteria
PLS-DA	Partial least squares discriminant analysis
PRRs	Pattern recognition receptors
Q-orbi-MS	Quadrupole-orbitrap-MS
Q-TOF-MS	Quadrupole-time-of-flight-MS
RLK	Receptor-like protein kinases
RLP	Receptor-like proteins

ROC	Receiver operator characteristic
ROS	Reactive oxygen species
RP	Reversed-phase
rRNA	Ribosomal ribonucleic acid
SA	Salicylic acid
SAG	Salicylic acid 2-O-beta-D-glucoside
SAR	Systemic acquired resistance
SCA	Simultaneous component analysis
Sd-RLKs	S-domain family of receptor-like kinases
SDS	Sodium dodecyl sulphate
SnA	Sinapoyl alcohol.
ST	Sweet/NS 5655
SVM	Support vector machine
UHPLC	Ultrahigh-performance liquid chromatographic
VIP	Variable importance in projection
VRM	Vitexin 2”-*O*-rhamnoside 7-*O*-methyltransferase
ZR	Zeatin riboside

List of Units

×*g*	Times gravity
°C	Degree Celsius
µL	Microlitre
µm	Micrometre
µM	Micromolar
µmol m^{-2} s^{-1}	Micromoles (Einstein's) per square meter per second
cm	Centimetre
d	Days
d.p.i.	Days post-inoculation
Da	Dalton
eV	Electron Volt
g	Grams
h	Hour
h.p.i.	Hours post-inoculation
kDa	Kilodalton
kV	Kilovolt
L	Litre
L h^{-1}	Litre per hour
M	Molar
mA	Milliamperes
mg	Milligrams
mg/L	Milligram per litre
min	Minutes
mL	Millilitre
mL/min	Millilitre per minute
mm	Millimetre
pg/mL	Picogram per millilitre
rpm	Revolutions per minute
Rt	Retention time
s	Seconds
V	Volts
v/v	Volume per volume
w/v	Weight per volume

List of Figures

Chapter 2

Chapter 3

Chapter 4

Chapter 5

List of Tables

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Summary

Metabolomics, the youngest sibling in the family of omics fields, has become an indispensable tool in studying plant biology. Considering that the metabolome is expectedly found to be sensitive to perturbations in both metabolic fluxes and enzyme activity, metabolomics provides insights into the physiological state and biological activities of an organism as influenced by changes in gene expression, protein function modulation and environmental cues. In plant science studies, the coordinated regulatory mechanisms underlying the immune responses of a biological system to biotic stresses can therefore be investigated by untargeted metabolomics approaches. The use of advanced analytical platforms such as LC-MS in untargeted plant metabolomics approaches facilitates a comprehensive measurement of metabolites, spanning an array of classes of these small-molecules. Such analyses, complemented with data analysis methodologies, thus reveal the molecular dynamics of the plant defence responses as well as biomakers associated with resistance state to an environmental stress. The capacity of a plant to launch an effective defensive state depends on the ability to perceive the pathogen presence (*via* MAMP perception) and timeous defence response activation. Upon pathogen detection, plant hormones such as the salicylates and jasmonates play key roles (working synergistically or antagonistically) to activate an array of highly regulated and coordinated defence events, involving a reprogramming of the metabolome, reflected through activation and changes in defence-related secondary metabolites and precursors for cell wall reinforcement.

Thus, to investigate biochemical processes and molecular mechanisms underlying defence responses in *Sorghum bicolor* (cvs NS 5511 and NS 5655) to the bacterial infection, by the pathogen *Burkholderia andropogonis,* a non-targeted metabolomics study based on LC-MS was conducted so as to unravel the metabolic signatures associated with the time-dependent and cultivar-specific host responses. The study was designed to comprise two major components: (i) sorghum leaves tissues infected with *B. andropogonis*, and (ii) sorghum cell suspension treated with the MAMP, LPS, isolated from the pathogen *B. andropogonis* ($LPS_{B. andr}$). To carry out the first study, *S. bicolor* plants at the four-leaf stage were treated with a suspension of *B. andropogonis*, and the infection monitored over a period of nine days (1, 3, 5, 7 and 9 d.p.i.). In the second study, LPS isolated from *B. andropogonis* was used to elicit

suspension-cultured cells and designed to monitor metabolic changes over time (0, 12, 18, 24 and 30 h.p.i.). Metabolites from the two biological systems were extracted with methanol and analysed on an UHPLC-QTOF-MS system. Raw data obtained thereof were processed and multivariate statistical analyses performed to facilitate the extraction of information from these complex data, and the identification of important biomarkers that define the host responses to the treatment. Annotation and biological interpretation of chemometrically selected metabolic signatures provided insightful description of cellular events occurring in the abovementioned interactions, specifically the host responses to the bacterial infection in both cases.

A disease severity-rating index, based on symptom evaluation, marked the onset and progression of bacterial infection in *S. bicolor* plants. The NS 5511 cv displayed delayed signs of wilting and lesion progression compared to the NS 5655 cv, indicative of enhanced resistance. Metabolomic analyses revealed that *B. andropogonis* triggered differential metabolic changes over time in the two *S. bicolor* cvs. These alterations could be visually assessed and infographically displayed by mass chromatograms. Furthermore, chemometric methods, such as principal component analyses (PCA), depicted such metabolic changes through sample groupings in PCA scores space: specifically time-related and cultivar-related metabolic changes. Metabolic 'stamps', explaining these measured and observed changes, span a wide range of the metabolome and include phytohormones, fatty acids, flavonoids and hydroxycinnamic acids. This metabolic reprogramming characterises the sorghum responses to the bacterial infection. Qualitatively, the two cvs responded in a similar manner and employed the same class of compounds associated with primary- and mostly secondary metabolism. However, NS 5511 accumulated crucial defence-related metabolites earlier and at elevated levels compared to NS 5655, explaining thus its resistant phenotype. Furthermore, results from the cell suspension study showed that purified $LPS_{B.\ andr.}$ triggered differential changes in the endo- and exometabolomes of *S. bicolor* cells over time, thus leading to variation in primary - and secondary metabolite biosynthesis.

Relative quantification revealed significant accumulation of various metabolites following treatment, thus suggesting defence-related roles played by these compounds. Of these classes of metabolites, flavonoids (such as apigenin and related glycosides), hydroxycinnamic derivatives (such as 4-coumaroylquinic acid, 3-feruloylquinic acid and sinapoyl alcohol) and fatty acids (such as the oxylipins, 15-hydroxylinoleic acid and 9,12,13-trihydroxy-10-octadecenoic acid), demonstrated to be important in the defence arsenal of sorghum plants.

Some interesting apigenin glycosides, namely vitexin (and related conjugates) and vicenin (1, 2 and 3), were identified for the first time in sorghum extracts. The relative levels and abundance of these metabolites, detected following infection, suggested their involvement in sorghum defence to bacterial infection. In cultured cells, flavonoids, hydroxycinnamic acids and fatty acids were also found to be a significant component of the 'defensome'. The fatty acid levels, particularly the oxylipins, dihydroxy-octadecadienoic acid, trihydroxy-octadecadienoic acid I and trihydroxy-octadecadienoic acid II, were evidently significant. Accumulation of phytohormones, which include salicylates and jasmonates, as well as hormone-responsive downstream metabolites such as phenylpropanoids, indicated that the various metabolic pathways were involved in defence responses. Interestingly, the various annotated metabolite classes reflected a significant diversity and extensive adaptive capabilities of sorghum in the event of stress.

Thus, the results in the plant leaf tissue and cell culture systems demonstrated metabolic reprogramming in *S. bicolor* plants and cultured cells following infection with *B. andropogonis* and treatment with LPS, respectively. This was marked by the defence arsenal in both systems spanning of range of classes of defence-related metabolites, of which accumulation was orchestrated by different plant hormones working synergistically or antagonistically to establish an enhanced defensive state. The early phenylpropanoid metabolic pathway (phenylalanine to hydroxycinnamates) and the late flavonoid pathway were found to be central in *S. bicolor* plant defence against *B. andropogonis*. In addition, metabolites from branches of the later flavonoid pathway such as the apigenin derivatives, vitexin and vicenin, not previously identified in sorghum, were demonstrated to be involved in *S. bicolor* plant defence, as highlighted by the multivariate statistical analyses. Based on disease severity rating of the cultivars and metabolomic analyses, NS 5511 cv (BT) demonstrated to have an enhanced capacity for inducible defence compared to NS 5655 cv (ST). Additionally, the results obtained in the cell culture study confirms that $LPS_{B.\ andr.}$ is perceived by *S. bicolor* to result in the triggering of defence-related metabolic reprogramming in sorghum. These results also revealed secretion of defence-related metabolites into the extracellular milieu by *S. bicolor* cells. The results from this study provide insightful biochemical description of sorghum responses to bacterial infection. The study contributes thus to ongoing efforts to understand molecular mechanisms underlying plant responses to biotic stresses.

Chapter 1: General Introduction

1.1. Justification of the study

There is an evident and rapid expansion of the human population, which is estimated to rise to 10 billion people by the year 2050 from the current 7 billion. The agricultural sector is consequently faced with pressure to substantially boost crop production to ensure food security. The highly productive agricultural and economically important crop *Sorghum bicolor* (referred to as sorghum hereafter), has a vast potential of contributing significantly to sustaining the expanding world population, *i.e*. providing food security in the future. Sorghum, a dry land crop, has the ability to thrive under different environmental conditions, especially harsh climates, and has thus been listed amongst the most productive agricultural crops. This crop is, however, greatly challenged by pathogen (biotic stressor) attack, which has led to the decrease in sorghum production over the past years. Due to the devastating crop/yield losses caused by pathogen attack, such stressors have been itemised as a prominent problem in crop production globally. In this view, there is a pressing need to reduce the use of chemical pesticides when dealing with pathogen challenge in crop production – because of the detrimental effects on the environment. Thus, improved and sustainable means to overcome such challenges, enhance crop resistance and increase crop/sorghum production are required. An understanding of the molecular mechanisms underlying sorghum–pathogen interactions will therefore be crucial in providing insights and useful knowledge in developing crop protection strategies through the exploitation of the plant's natural defence mechanisms.

1.2. General introduction to the study

Sorghum is a multipurpose cereal crop, and is also more cost-effective to produce relative to other cereals. This is owing to the crop's ability to perform better under environmental stresses, ultimately providing substantial yields (Awika & Rooney, 2004; Dicko *et al.*, 2006; Poloni & Schirawski, 2014). Sorghum ranks as the fifth most important world cereal, exceeded only by wheat, rice, corn and barley (Ritter *et al.*, 2007; Paterson, 2008; Dube *et al.*, 2010; Mwadalu & Mwangi, 2013; Amelework *et al.*, 2016); and is the second most important cereal crop in semi-arid tropics (Amelework *et al.*, 2015). Moreover, the crop is amongst the most productive

agricultural crops, making it a principal (staple) source of food for millions of people in over 30 countries globally (Dube *et al.*, 2010), and with a record of around 300 million people sustained in Sub-Saharan Africa (Amelework *et al.*, 2015, 2016). Other uses of sorghum include animal feed (primarily in developed countries), and energy (fuel; ethanol) -, alcoholic beverages -, consumable oils - and fertiliser production, amongst others (Owuama, 1999; Tuinstra, 2008; Guo *et al.*, 2011; Tari *et al.*, 2012; Poloni & Schirawski, 2014).

Regions other than Africa also principally relying on sorghum include Central America, some parts of Asia, and the Middle East (Dicko *et al.*, 2006; Taylor *et al.*, 2006; Ritter *et al.*, 2007; Tuinstra, 2008; Tari *et al.*, 2012). Thus, a major portion of the global food supply is provided by cereal crops such as sorghum (Du Fall & Solomon, 2011; Balmer *et al.*, 2013). More than 60 million tonnes of sorghum yield are produced annually on a global scale (Taylor, 2003; Njiru, 2010), with the United States, Mexico, Nigeria, Sudan and India being the top 5 global producers (listed in order from 1 to 5) in 2016 (http://www.sorghumafrica.com/news).

Furthermore, sorghum has various health benefits which have led to its recently gained attention at different levels and sectors. The health benefits arise from the crop's naturally rich and diverse phytochemical composition, nutritionally vital proteins and micronutrients amongst other bioactive compounds (Awika & Rooney, 2004; Belton & Taylor, 2004; Taylor *et al.*, 2006). Some of the reported beneficial phytochemicals include phenolic acids and anthocyanins, exhibiting high antioxidant activity towards prevention or reduction of oxidative stress, thus subsequently reducing the risk of developing chronic diseases such as diabetes and certain types of cancer, respectively (Awika & Rooney, 2004; Awika, 2011; Stefoska-Needham *et al.*, 2015; Kang *et al.*, 2016). Sorghum phytosterols and policosanols have also been reported to have cholesterol lowering properties and, as such, play a role in reducing cardiovascular disease risk. Health-promoting properties of sorghum have been clearly summarised by Taylor *et al.* (2014). In addition, this crop has also become very important for the food industry due to its gluten-free attribute and thus has captured attention of scientists and the public for use as an alternative food crop for coeliac disease-suffering - and gluten-intolerant individuals (Taylor *et al.*, 2014; Stefoska-Needham *et al.*, 2015; Mathur *et al.*, 2017).

Thus, sorghum is proving to be important in providing food security and its nutritionally rich phytochemicals are important for communities at health risks (Taylor *et al.*, 2014). However, sorghum is attacked by a wide range of pathogens affecting its production (Poloni &

Schirawski, 2014). Pathogen attack on crops is a major agro-economical concern due to the dramatic yield-losses that arise therefrom (Du Fall & Solomon, 2011; Ahuja *et al.*, 2012; Balmer *et al.*, 2013; Ranf *et al.*, 2015). In particular, the bacterial pathogen *Burkholderia andropogonis* causes bacterial leaf stripe, which is one of the three major bacterial diseases affecting sorghum (Navi *et al.*, 2002; Paganin *et al.*, 2011).

Naturally, for the defence against biotic stress, plants as sessile organisms, rely on evolutionarily dynamic innate immunity involving constitutive and inducible defence mechanisms to eliminate/limit such pathogen invasions (Anjum *et al.*, 2013; Meyer *et al.*, 2015). The recognition of conserved features known as microbe- or pathogen-associated molecular patterns (MAMPs) on the pathogen by plant receptors activates signalling cascades. This leads to defence-related cellular and molecular events such as the production of reactive oxygen species (ROS) (Du Fall & Solomon, 2011; Atkinson & Urwin, 2012), expression of defence-related genes (Klemptner *et al.*, 2014), colonised tissue lignification (Anjum *et al.*, 2013; Poloni & Schirawski, 2014) and anti-microbial compounds production (Meyer *et al.*, 2015). Thus, there is enhancement of physical, enzymatic, and chemical defences. Chemical defences (of particular interest in this metabolomic study) – forming part of the plant's important defence repertoire – include defence-related secondary metabolites (Zvereva & Pooggin, 2012; Poloni & Schirawski, 2014; Andolfo & Ercolano, 2015; Gao *et al.*, 2015). The outcome of these defence responses is greatly influenced by three factors *i.e.* ability of a plant to perceive the pathogen presence (MAMP perception), defence response activation and the pathogen's ability to suppress the immune system of the plant (Pieterse *et al.*, 2009; Pritchard & Birch, 2011).

Metabolomics, the qualitative and quantitative analysis of small molecules (the metabolome) in a biological system is an indispensable tool in studying biological systems (Goodacre *et al.*, 2004; Bino *et al.*, 2004; Hall, 2006; Misra *et al.*, 2017). As the metabolome is a representation of the definitive cellular phenotype influenced by gene expression perturbations, protein function modulation and environmental cues, a metabolomics approach (recent '-omics' approach) can give insights into the physiological state and biological activities of an organism (Goodacre *et al.*, 2004; Verpoorte *et al.*, 2008; Allwood *et al.*, 2011; Tugizimana *et al.*, 2013). Hence, this omics approach can be used to assess the cellular changes in plants induced by exogenous factors, for example, biotic stress such as pathogen attack – with the metabolic changes best reflecting the cellular events occurring as a result of interactions between the plant

and pathogen (Bhalla *et al.*, 2005; Hall, 2006; Allwood *et al.*, 2008; Hall *et al.*, 2008; Misra *et al.*, 2017). Furthermore, metabolomics through metabolic profiling is a useful approach in determining key infection, resistance and plant response biomarkers in plant–pathogen studies as well as in revealing novel pathways involved in stress responses (Cuperlovic-Culf *et al.*, 2016; Kumari & Parida, 2018).

The current study thus focuses on understanding the molecular mechanisms involved in *S. bicolor–B. andropogonis* interactions, employing a metabolomics approach. Two cultivars of sorghum, differing in disease susceptibility/resistance, were used and the metabolic changes were monitored over time following infection treatment of 4-week old sorghum plants with *B. andropogonis*. To the best of our knowledge, no studies to date have reported on the metabolic reprogramming occurring in sorghum following *B. andropogonis* bacterial infection.

1.3. Hypothesis

The metabolome of sorghum cultivars differing in disease susceptibility/resistance following *B. andropogonis* infection results in distinguishable metabolite fluctuations which can be investigated by liquid chromatography and mass spectrometry (LC-MS) using a non-targeted metabolomics approach.

To address this hypothesis the following aims and objectives were formulated.

1.4. Aim

- To investigate the metabolic changes in *S. bicolor* cultivars (NS 5511 and NS 5655) following infection by the bacterial pathogen, *B. andropogonis,* using a non-targeted metabolomics approach.

- To investigate LPS isolated and purified from *B. andropogonis* (a microbe/pathogen associated molecular pattern – MAMP) as one of the instrumental factors triggering metabolomic reprograming in sorghum.

1.5. Objectives

- Infection of sorghum cultivars differing in disease susceptibility with *B. andropogonis* and harvesting leaf tissue at specific time intervals following challenge.
- Isolation and purification of lipopolysaccharides (LPS, a surface-located MAMP) from *B. andropogonis.*
- Elicitation of sorghum cell suspensions with purified LPS.
- Extraction of metabolites from treated and non-treated plants as well as cultured suspension-grown cells.
- Analysis of the extracted metabolites on a liquid chromatography coupled to mass spectrometry (LC-MS) platform.
- Chemometric analyses of the data and annotation of the significant biomarkers (metabolites) synthesised in sorghum plants in response to bacterial infection and in sorghum cells in response to LPS elicitation.
- Comparison of the induced defence responses in differentiated leaf tissue and undifferentiated cells in culture.
- Biological interpretation of the acquired results (linking the results to the biological question).

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Chapter 2: Literature Overview

2.1. Sorghum plant – a food crop: an overview

Sorghum is a self-pollinating monocotyledonous cereal crop (Paterson, 2008; Hartz *et al.*, 2009; Stefoska-Needham *et al.*, 2015) with a small diploid genome size is ~730 Mbp (mega base pairs, with haploid chromosome number of 10) (Swigoňová *et al.*, 2004; Kim *et al.*, 2005; Paterson, 2008; Paterson *et al.*, 2009). The sorghum plant is a the tropical crop native to Africa (Tari *et al.*, 2012) and belongs to the family of Poaceae (Gramineae), tribe of Andropogoneae and genus *Sorghum* in which both cultivated and wild species exist. In this genus, *Sorghum bicolor* (L.) Moench is the species that is principally cultivated; hence, the name sorghum is generally used to refer to *Sorghum bicolor* (the same is thus used throughout the Chapters in this dissertation) (Owuama, 1999; Dicko *et al.*, 2006; Ritter *et al.*, 2007; Tari *et al.*, 2012). Intriguing qualities of the cereal crop making it of particular interest and value include drought tolerance (Liu *et al.*, 2010), the ability to withstand harsh climates (*e.g.* high temperatures) and waterlogging periods (Lara & Andreo, 2011; Tari *et al.*, 2012; Muui *et al*, 2013; Mwadalu & Mwangi, 2013), the crop's naturally rich and diverse phytochemical composition (Awika, 2011; Kang *et al.*, 2016) as well as the nutritional potential (Taylor *et al.*, 2014).

The continuous increase in the human population recorded annually, imposes pressure on agricultural crop production systems. Sorghum production, on the other hand, has great potential of providing food security in the future because of its adaptation to various environmental conditions. However, in the past 30 years significant decline in sorghum production has been witnessed (Paterson, 2008; Taylor *et al.*, 2014; Wise, 2014). This is due to challenges imposed by abiotic and biotic stresses – like all other plants in nature (Du Fall & Solomon, 2011; Denancé *et al.*, 2013), which will be discussed in **section 2.1.1**. The severity of some of the factors on sorghum production, however, may vary with the plant's growth stage and with the region of sorghum cultivation (Rao, 2004; du Plessis, 2008; Amelework *et al.*, 2016; Knott *et al.*, 2016).

2.1.1. Stress factors negatively acting on sorghum production

Both abiotic and biotic stresses have deleterious effects on sorghum production (du Plessis, 2008; Hartz *et al.*, 2009; Tari *et al.*, 2012; Ciampitti *et al.*, 2014). However, focus of the current study is on biotic factors. The latter, including weeds, pests (herbivores) and pathogens negatively affect sorghum production, sometimes to alarming extents (Kochenower *et al.*, 2010; Guo *et al.*, 2011; Ciampitti *et al.*, 2014; Knott *et al.*, 2016). Viral, bacterial or fungal pathogens cause various diseases which, in turn, affect the production of sorghum – leading to poor yield quality and quantity (economic losses) (Kosambo-ayoo *et al.*, 2011; Poloni & Schirawski, 2014). Of these, pathogen attack is one of the leading stressors greatly impacting on sorghum production globally. A summary of some bacterial and fungal diseases affecting sorghum production is listed in **Table 2.1**. There are extensive studies on sorghum–fungal pathogen interactions and disease that arise therefrom. However, reports on sorghum–bacterial pathogen interactions and bacterial diseases affecting sorghum are limited; hence, there is a poor understanding on the aetiology and epidemiology of these diseases (Claflin *et al.*, 1992; Little *et al.*, 2012). Thus, the current study intends to expand on the current knowledge regarding sorghum–bacterial pathogen interactions and sorghum bacterial diseases.

Understanding the molecular mechanisms underlying sorghum–bacterial pathogen interactions is imperatively essential, as it would provide insights and useful knowledge in developing strategies to aid sorghum plants to adapt and defend against continuously evolving bacterial pathogens (Andolfo & Ercolano, 2015). Some studies have reported that sorghum–bacterial pathogen interactions entails the colonisation of parenchymatous plant tissue (Bagsic *et al.*, 1995) and that apigeninidin, one of the 3-deoxyanthocyanidin phytoalexins (defence secondary metabolites) can inhibit bacterial growth. This compound, known to inhibit fungal growth, is thus not pathogen specific (Poloni & Schirawski, 2014). Although such studies have provided substantial knowledge and advanced our understanding of defence responses arising from sorghum–bacterial pathogen interactions, such reports are just the tip of an iceberg. Comprehensive functional and mechanistic descriptions of molecular communication and metabolic regulation that govern sorghum–bacterial pathogen interactions, are still limited. Thus, the current study is an untargeted metabolomics approach to uncover the molecular signatures that define biochemical processes involved in sorghum responses to bacterial infection by the pathogen *Burkholderia andropogonis*.

Table 2.1: A summary of the common bacterial and fungal diseases affecting *Sorghum bicolor*.

Disease	Causal agent	Pathogen class	Symptoms/ characteristics	Source
Bacterial leaf stripe	*Burkholderia andropogonis*	Bacteria	Red, tan, yellow or purple, narrow elongated linear lesions (lesion colour depends on the response and/ or genotype of affected plant).	Akhtar, 1985; Claflin *et al*., 1992; Navi *et al*., 2002; Ramundo & Claflin, 2005; Knott *et al*., 2016
Bacterial leaf streak	*Xanthomonas campestris* pv. *holcicola*	Bacteria	Short red-brown streaks that later elongate developing long tan centres with red narrow margins.	Claflin *et al*., 1992; Navi *et al*., 2002; Knott *et al*., 2016
Bacterial leaf spot	*Pseudomonas syringae*	Bacteria	Small tan spots encircled by dark margins.	Claflin *et al*., 1992; Navi *et al*., 2002; TeBeest *et al*., 2004; Knott *et al*., 2016
Anthracnose	*Colletotrichum sublineolum*	Fungi	Coalescent elongated tan to red lesions. Acervuli at the centre of lesion as the fungus sporulates (symptoms varying depending on the host, pathogen and environmental interactions).	Perfect *et al*., 1999; Marley *et al*., 2001; TeBeest *et al*., 2004; Ibraheem *et al*., 2010; Liu *et al*., 2010; Were & Ochuodho, 2012
Leaf blight	*Exserohilum turcicum*	Fungi	Start as small red-tan spots which can develop into grey centred long, elliptical lesions bordered with tan to red margins. (colour dependent on cultivar)	TeBeest *et al*., 2004; Knott *et al*., 2016
Charcoal rot	*Macrophomina phaseolina*	Fungi	Lodging of the affected plant with parched and stringy zones present on the stem, close to the bend together with black sclerotium appearances.	Claflin *et al*., 1992; TeBeest *et al*., 2004; Knott *et al*., 2016
Rust	*Puccinia purpurea*	Fungi	Spots, purple or red appearing on upper and lower leaf surfaces which later develop into brown- dark red uredinia in susceptible cultivars (uredinia is parallel to leaf veins).	Thakur *et al*., 2007; Knott *et al*., 2016
Downy Mildew	*Perono-sclerospora sorghi*	Fungi	Infected plants exhibit chlorotic and stunted appearances with green and white stripes developing on emerging leaves. White and downy growth of fungal spores on leaf surface. Leaves eventually shred.	Craig & Odvody, 1992; Thakur *et al*., 2007; Kochenower *et al*., 2010; Ciampitti *et al*., 2014

2.2. *Burkholderia andropogonis* – causal agent of one of the major sorghum bacterial diseases

The genus *Burkholderia* is comprised of many species that occupy diverse ecological niches, playing various roles (Stoyanova *et al.*, 2007; Bournaud *et al.*, 2013; Estrada-De Los Santos *et al.*, 2013; Palleroni, 2015). Thus far, more than 95 species have been identified to fall under this genus (Lopes-Santos *et al.*, 2017). The first *Burkholderia* species were identified in 1942 by Walter Burkholder (Compant *et al.*, 2008). Eventually the genus *Burkholderia* (named after W. Burkholder) was then established by Yabuuchi and colleagues in 1992 for the specific reason of accommodating seven species that belonged to group II of *Pseudomonas* rRNA. Later, additions were made to the genus to include more species (Yabuuchi *et al.*, 1992; Palleroni, 2015; Eberl & Vandamme, 2016; Choma *et al.*, 2017). Members of this genus are β-proteobacteria known to affect humans, animals as well as plants (Paganin *et al.*, 2011; Bournaud *et al.*, 2013). Plant-associated *Burkholderia* species are either pathogenic or non-pathogenic (beneficial or neutral) to the host plants. Phytopathogenic *Burkholderia* species are causative agents of various plant diseases and these include *B. andropogonis*, *B. cepacia*, *B. glumae*, *B. caryophylli*, *B. plantarii* and *B. gladioli* to name but a few. Of these pathogenic species *B. andropogonis* is of interest in this study (Estrada-De Los Santos *et al.*, 2001, 2013; Compant *et al.*, 2008; Paganin *et al.*, 2011; Eberl & Vandamme, 2016).

B. andropogonis, the causal agent of sorghum leaf stripe disease (Ramundo & Claflin, 2005; Paganin *et al.*, 2011; Palleroni, 2015), was first defined as an important pathogen for the disease by Smith in 1911 (Coenye *et al.*, 2001; Duan *et al.*, 2009; Lopes-Santos *et al.*, 2015). This bacterial pathogen, formerly known as *Pseudomonas andropogonis*, was reclassified to the genus *Burkholderia* by Gillis *et al.* (1995), following DNA-rRNA hybridisation studies (Gillis *et al.* 1995; Coenye *et al.*, 2001; Duan *et al.*, 2009; Lopes-Santos *et al.*, 2015). Also, following chemotaxonomic, phenotypic and genotypic confirmation studies, *Pseudomonas woodsii*, which was also previously regarded as synonym for *B. andropogonis*, was concluded to be the same species as *B. andropogonis* (Gillis *et al.*, 1995; Coenye *et al.*, 2001; Duan *et al.*, 2009). This pathogen is a Gram-negative, non-spore forming, aerobic, soil bacterium of about 0.5 × 1.5 µm in size, lacks fimbriae, slim rod shaped and a single polar sheathed flagellum per cell that enables mobility (**Figure 2.1**). In addition, *B. andropogonis* produces rhizobitoxine which can cause foliar chlorosis in host plants (Claflin *et al.*, 1992; Bagsic *et al.*, 1995; Cother *et al.*, 2004; Lopes-Santos *et al.*, 2015; Palleroni, 2015). The production of rhizobitoxine and single

polar flagellum are distinctive features of the pathogen (Lopes-Santos *et al.*, 2015). *B. andropogonis* species have a diverse and extensive geographical dispersion, and host range (Bagsic *et al*., 1995; Duan *et al.*, 2009; Lopes-Santos *et al.*, 2015).

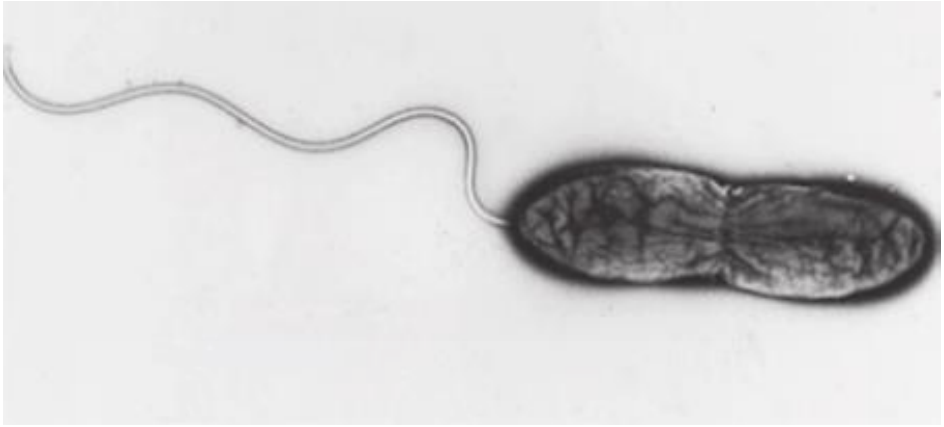

Figure 2.1: A representation of *Burkholderia andropogonis* morphology. The Gram-negative bacterium is slim and rod shaped, and has a polar sheathed flagellum attached. The flagellum enables bacterial mobility. Only one flagellum is attached per each bacterial cell. Approximate size of the bacterial cell is 0.5 × 1.5 µm. No fimbriae and prosthecae production associated to the bacterium has been recorded (taken from Palleroni, 2015).

2.2.1. Bacterial leaf stripe symptoms

Bacterial leaf stripe is amongst the three major bacterial diseases of economic importance affecting sorghum; the other two diseases being bacterial streak *(Xanthomonas campestris pv holcicola*) and bacterial spot (*Pseudomonas syringae*) (Claflin *et al*., 1992; Navi *et al.*, 2002). Symptoms of bacterial leaf stripe occur primarily on leaves; these include linear lesions red, yellow, tan or purple in colour (**Figure 2.2**) running along the veins due to invasion of the leaf tissue. The lesion colour typically depends on the affected plant's response or colour (Claflin *et al*., 1992; Ramundo & Claflin, 2005) and the shape of lesions is usually narrow and elongated, appearing first on lower leaves (Knott *et al*., 2007; Cunfer, 2015). In addition, bacterial cell exudates can also be noticed on the underside of the infected leaves (Claflin *et al*., 1992). According to Bagsic *et al*. (1995), the leaf stripes, streaks or spots that appear on host plants result from the invasion of parenchymatous tissues by the pathogen.

Generally, bacterial pathogens gain entry into a host plant through natural openings (*e.g*. stomata), wounds, and abrasions or with the aid of feeding insects (Vidaver & Lambrecht, 2004). It can thus, be suggested that the infection process by *B. andropogonis* in an unwounded

plant likely begins when the pathogen gains entry by swimming through the stomata (since both adaxial and abaxial leaf surfaces of sorghum have stomata) with the aid of its flagellum (Taylor, 2003; Mwadalu & Mwangi, 2013; Palleroni, 2015). However, the mechanism by which the lesions develop on sorghum hosts in response to this invasion has not been detailed, to this date (if it is as a result of accumulation of defence-related compounds or death of the infected cells or other events). Other parts (secondary parts) of the plant affected by *B. andropogonis* are stems, flower buds, calyxes (Bagsic *et al.*, 1995), peduncle, stalk interior and seeds (Claflin *et al.*, 1992). Duan *et al.* (2009) reported that infection of host plants by *B. andropogonis* also induces plant tissue chlorosis or necrosis *via* the production of non-host-specific toxins. It is noteworthy that the symptoms of bacterial leaf stripe often resemble bacterial streak symptoms and similarities between these and those of numerous fungal diseases can also be noticed and, thus, should not be mistaken for each other (Claflin *et al.*, 1992).

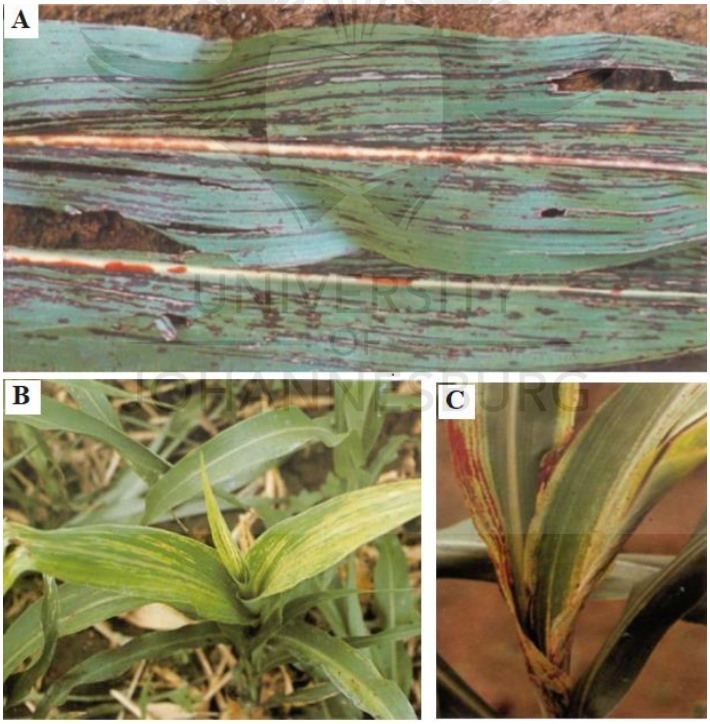

Figure 2.2: Bacterial leaf stripe symptoms noticed on field sorghum crops. The colour of the lesions depends on the reaction of the host plant toward the bacterial infection, with colours ranging from red, yellow, tan or purple (as seen in **A**, **B** and **C**). Bacterial stripe symptoms are mostly dominant on the leaves but also occur on other parts of the plant (taken from Williams *et al.*, 1978).

The use of chemicals such as bactericides and fungicides to protect crops against pathogen attack is not only toxic to the environment and human health (as some of these chemicals are not biodegradable) but is also expensive. For these reasons, alternative ways to eradicate

pathogens are currently being investigated (Muriithi & Claflin, 1997; Aktar *et al.*, 2009; Mhlongo, 2015). Enhancement/manipulation of plants' natural defence mechanisms such as enhanced production of defence-related secondary metabolites, as an alternative approach to fight pathogen attack, has shown to be functional in increasing host resistance (Morrissey & Osbourn, 1999; Hernández *et al.*, 2009; Jeet *et al.*, 2014; Ibraheem *et al.*, 2015; Meyer *et al.*, 2015). Thus, the present study, contributing to this scientific endeavour, intends to profile differential defence-related metabolites deployed by sorghum in response to the bacterial pathogen attack.

2.3. An overview of plant defence mechanisms

2.3.1. Innate immune response in plants – a general description

Plants are continuously exposed to an array of pathogens which can either be host specific or can affect a wide range of hosts. These pathogens employ different lifestyles, *e.g*. biotrophic, necrotrophic or hemibiotrophic (Niks & Marcel, 2009; Balmer *et al.*, 2013; Andolfo & Ercolano, 2015; Bigeard *et al.*, 2015; Meyer *et al.*, 2015) and, as such, knowledge on the nature of the interaction between the host and the pathogen facilitates in understanding the defence responses employed by the plant. In biotrophic interactions, the pathogen poses less harm and extracts nutrients for survival, without killing its host (Andolfo & Ercolano, 2015; Gao *et al.*, 2015; Spanu & Panstruga, 2017). On the other hand, in necrotrophic interactions the pathogen destroys its host through production of toxins and cell wall-degrading enzymes, and eventually feeds on nutrients released by dead host cells (Mengiste, 2012; Andolfo & Ercolano, 2015; Shigenaga & Argueso, 2016). In cases where the pathogen adopts both lifestyles, starting as a biotroph and later switching to necrotrophy, this is referred to as a hemibiotrophic interaction (Mengiste, 2012; Spanu & Panstruga, 2017).

As immobile organisms, lacking a circulatory system and specialised immune cells to protect themselves, plants employ several layers of defence mechanisms (preformed and inducible) to alleviate the potential damage by pathogens (Oostendorp *et al.*, 2001; Cheynier *et al.*, 2013; Andolfo & Ercolano, 2015; Gao *et al.*, 2015; Meyer *et al.*, 2015). Preformed defences provide physical and chemical barriers that prevent or minimise the pathogen invasion (Yang *et al.*, 1997; Mithöfer & Boland, 2012). For instance, physical barriers, such as rigid cell walls, waxes and cuticles, as well as spikes and thorns provide protection against attacking herbivores and

insects (Bektas & Eulgem, 2014; Bigeard *et al.*, 2015). In addition to physical barriers, preformed defences include chemical deterrents that involve constitutively expressed defence-related metabolites known as phytoanticipins (see **section 2.3.4.2**). The latter are present in healthy plants (prior to a pathogen attack), in active forms or as inactive precursors which are activated upon response to tissue damage (Osbourn, 1996; Mithöfer & Boland, 2012; Klemptner *et al.*, 2014; Bigeard *et al.*, 2015). Inducible defences are those activated in the plant following pathogen attack, with the aim to further limit pathogen proliferation. These include chemical defences such as phytoalexin, *de novo* biosynthesised upon pathogen stress (see **section 2.3.4.2**) (Mazid *et al.*, 2011; Balmer *et al.*, 2013).

Plant innate immune responses hinder attempted invasion and/or limit infection after invasion (basal resistance). These defence mechanisms have been represented as a 'zig-zag model' and include perception of non-self (by immune receptors) and activation of signalling events that lead to cellular reprogramming (**Figure 2.3)** (Jones & Dangl, 2006; Bari & Jones, 2009; Niks & Marcel, 2009; Zvereva & Pooggin, 2012). Following attempted invasion, the first reaction involves recognition of conserved invariant structures of microbial origin (known as microbe- or pathogen-associated molecular patterns, M/PAMPs) by pattern recognition receptors (PRRs). These receptors are located on the plant cell surface and are usually receptor-like protein kinases (RLK) / receptor-like proteins (RLP), *e.g.* members of receptor-like kinases of the leucine-rich repeat family (LRR-RKs) or S-domain family (Sd-RLKs). For intracellular stimuli, recognition is performed by nucleotide-binding (NB)-LRR receptors (Bittel & Robatzek, 2007; Tsuda & Katagiri, 2010; Bigeard *et al.*, 2015; Choi & Klessig, 2016). The direct interaction between PRRs and M/PAMPs, in turn, activates a complex set of responses known as M/PAMP-triggered immunity (M/PTI, **Figure 2.3**) (Niks & Marcel, 2009; Chen & Ronald, 2011; Maffei *et al.*, 2012; Zvereva & Pooggin, 2012; Bigeard *et al.*, 2015). In *Arabidopsis*, the N-terminus of the bacterial flagellin (flg22) from *P. syringae* is recognised by the flagellin-sensitive 2 (FLS2) receptor and, similarly, the EF-Tu receptor (EFR) recognises the bacterial elongation factor Tu (EF-Tu) through its elf18 epitope, thus demonstrating the PRR/MAMP interaction for triggering the chain of signalling events associated with M/PTI immunity (Ingle *et al.*, 2006; Shan *et al.*, 2008; Qi *et al.*, 2011; Bigeard *et al.*, 2015; Choi & Klessig, 2016).

M/PTI responses are also triggered by products from the host produced during or subsequent to the process of pathogen attack because of damage, termed damage- or danger-associated

molecular patterns (DAMPs). Both M/PAMPs and DAMPs appear to be undifferentiated by plants and the responses are comparable (Zvereva & Pooggin, 2012; Andolfo & Ercolano, 2015; Bigeard *et al.*, 2015; Choi & Klessig, 2016). Recently, the terms HAMPs (herbivore-associated molecular patterns) and NAMPs (nematode-associated molecular patterns) have been used to indicate compounds from herbivorous insects and nematodes, respectively, also perceived by plants (Maffei *et al.*, 2012; Choi & Klessig, 2016). The most known (and common) physiological response to DAMP/HAMP/MAMP perception is an increase in cytosolic Ca^{2+} concentration due to an influx from external stores or a release from internal storage compartments (Zvereva & Pooggin, 2012; Bigeard *et al.*, 2015; Monaghan *et al.*, 2015).

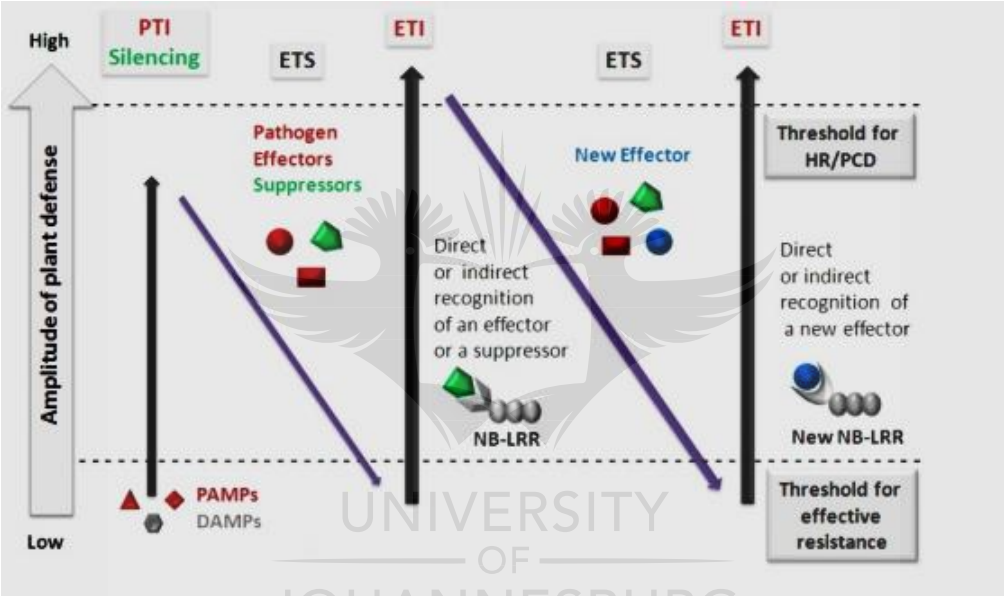

Figure 2.3: A zig-zag model representing the plant innate immune system. The first line of defence (**M/PAMP**-triggered immunity, **M/PTI**) activated to inhibit/limit pathogen infection is initiated upon recognition of **M/PAMPs** by pattern recognition receptors (**PRRs**). When pathogens succeed in supressing or evading **M/PTI** through the release of suppressors or effectors that interfere with **M/PTI,** effector-triggered susceptibility (**ETS**) arises. Recognition of a specific effector by a specific resistance (**R**) protein (*e.g.* nucleotide-binding –leucine rich repeats proteins **NB-LRR**) results in effector-triggered immunity (**ETI**). The intensity (amplitude) of the host response during ETI is greater than during M/PTI. Events such as the hypersensitive response (**HR**, a form of programmed cell death, **PCD**) take place at the site of infection, limiting the infection from spreading to other parts of the plant. Other events triggered by **ETI** are local acquired resistance (**LAR**) to contain the intruder at the infection site, and systemic acquired resistance (**SAR**), that provides immunity in other parts of the plant distant to the site of infection (adapted from Jones & Dangl 2006; Zvereva & Pooggin, 2012).

Some microbial pathogens may be well-adapted to their host and secrete effector molecules that can attenuate M/PTI, thus weakening the defence response which, in turn, can cause the plant to be susceptible to induced disease, known as effector-triggered susceptibility (ETS). The latter occurs if the effector molecules from the pathogen are not recognised by the host plant (**Figure 2.3**) (Bari & Jones, 2009; Niks & Marcel, 2009; Bigeard *et al.*, 2015). M/PTI responses ensure resistance to all types of pathogens, such as fungi or bacteria, through

increased ion influx across the plasma membrane, cell wall reinforcement as well as production of reactive-oxidative species (ROS) (Ingle *et al.*, 2006; Mejía-Teniente *et al.*, 2010; Návarová *et al.*, 2012; Zvereva & Pooggin, 2012). Although this response may limit the spread of virulent pathogens, it is insufficient to prevent disease development in certain cases (Bektas & Eulgem, 2014).

Direct or indirect recognition of highly variable effectors by host receptors, which are encoded by disease resistance (*R*)-genes, activates the second line of defence known as effector-triggered immunity (ETI). This mediates a gene-to-gene (also referred to as race-specific) resistance that is more specific, quicker and robust, as well as more prolonged than M/PTI (**Figure 2.3)**. The same (*R*)-gene can confer resistance to more than one microbe or different (*R*)-genes towards multiple microbes (Bari & Jones, 2009; Yin *et al.*, 2012; Zvereva & Pooggin, 2012; Bektas & Eulgem, 2014; Andolfo & Ercolano, 2015; Bigeard *et al.*, 2015). Due to its specificity, ETI exhibits a stronger immune response that provides efficient protection against virulent pathogens and is usually associated with the hypersensitive response (HR), where salicylic acid (SA) plays a vital role in activation. This reaction involves a programmed cell death (PCD) at the site of infection, thus preventing the spread of the pathogen to other parts of the plants (Ingle *et al.*, 2006; Ma, 2011; Zvereva & Pooggin, 2012; Bektas & Eulgem, 2014; Andolfo & Ercolano, 2015). The HR process is associated with the production of signalling molecules such as nitric oxide (NO) and ROS such as hydrogen peroxide, singlet oxygen and hydroxyl radicals (Ingle *et al.*, 2006; Ma, 2011).

The plant innate immune system also comprises various signalling pathways such as the mitogen-activated protein kinase (MAPK) cascade and signalling molecules (phytohormones) leading to expression of defence-related genes (*e.g.* phenylalanine ammonia lyase, PAL) and production of antimicrobial compounds (phytoalexins) (Benhamou, 1996; Yang *et al.*, 1997; Klessig *et al.*, 2000; Henry *et al.*, 2012) and allelopathic molecules (Bourgaud *et al.*, 2001; Zvereva & Pooggin, 2012). Both of these immunities (M/PTI and ETI) are transient, local responses and enhance resistance. Induced resistance (IR) can be expressed at a local level (LIR), limited to pathogen infected tissues and do not offer a long-lasting resistance. In contrast, systemic acquired resistance (SAR, **section 2.3.2**), dependent on long-range chemical signalling, has been shown to confer a long-lasting protection against a broad-range of pathogens in uninfected tissues (Chen & Ronald, 2011; Bektas & Eulgem, 2014).

2.3.2. Systemic acquired resistance (SAR), induced systemic resistance (ISR) and the role of phytohormones in plant defence

Plants can alert distal parts by sending signals to un-infected areas and induce a broad spectrum and long-lasting systemic form of resistance to secondary pathogens, SAR (Oostendorp *et al.*, 2001; Zhao *et al.*, 2005; Zvereva & Pooggin, 2012; Gao *et al.*, 2015). Also, plant interaction with soil microbes can lead to the induction of systemic resistance in distal parts termed induced systemic resistance (ISR). Both forms of resistance are effective against a wide range pathogens (**Figure 2.4**) (Oostendorp *et al.*, 2001; Van Der Ent *et al.*, 2009; Tenenboim & Brotman, 2016).

SAR responses may be induced by biological application of infectious agents (localised infection), or chemical inducers such as SA or SA analogues (Oostendorp *et al.*, 2001; Vlot *et al.*, 2009; Bektas & Eulgem, 2014). In plant–pathogen interactions SA is an important inducer of SAR, which is mainly mediated by this phytohormone and its methyl ester (MeSA). Following successful infection, the hormone rapidly accumulates at the infection site (usually effective for biotrophic pathogens) and then translocates to other parts of the plant, which results in a signal for activation of SAR and other enhanced defence mechanisms (Zhao *et al.*, 2005; Bari & Jones, 2009; Zvereva & Pooggin, 2012). Exogenous administration of SA induces expression of pathogenesis-related (*PR*) genes (such as *PR1*, *PR2* and *PR5*) that serves as a robust marker for SAR (Oostendorp *et al.*, 2001; Manosalva *et al.*, 2010; Conrath, 2011; Bektas & Eulgem, 2014). Other SAR signalling molecules includes azelaic acid, MeSA, jasmonates (JA, MeJA and ileu-JA) and diterpenoids such as dehydroabietic acid (Manosalva *et al.*, 2010; Henry *et al.*, 2012; Zvereva & Pooggin, 2012). Interestingly, SAR can be conveyed to the direct succeeding generation of progeny through chromatin structure modifications (Gao *et al.*, 2014).

On the other hand, ISR is a result of plant root colonisation by beneficial soil microbes (Goellner & Conrath, 2008; Badri *et al.*, 2009; Zamioudis & Pieterse, 2012). For example, non-pathogenic microbes such as plant growth-promoting rhizobacteria (PGPR; such as *Pseudomonas* spp. and *Bacillus* spp.) and symbiotic fungi (PGPF; *Trichoderma* spp.), have been shown to colonise plant roots, inducing ISR to protect the above-ground plant tissues against pathogens (Van Der Ent *et al.*, 2009; Zamioudis & Pieterse, 2012). The major regulators of ISR are jasmonic acid (JA) and ethylene (ET) (Oostendorp *et al.*, 2001; Goellner & Conrath, 2008). For instance, prior application of these two phytohormones, in plants such

as *Arabidopsis*, increases production of defensins (antimicrobial peptides), when inoculated with stressors such as *Alternaria* (Oostendorp *et al.*, 2001; Vlot *et al.*, 2009). This pre-exposure to eliciting agents such as JA or ET allows the plant to instigate a more prompt and intense defence responses to a subsequent attack, a concept described as pre-conditioning or plant priming (Denancé *et al.*, 2013; Mhlongo *et al.*, 2016; Tenenboim & Brotman, 2016).

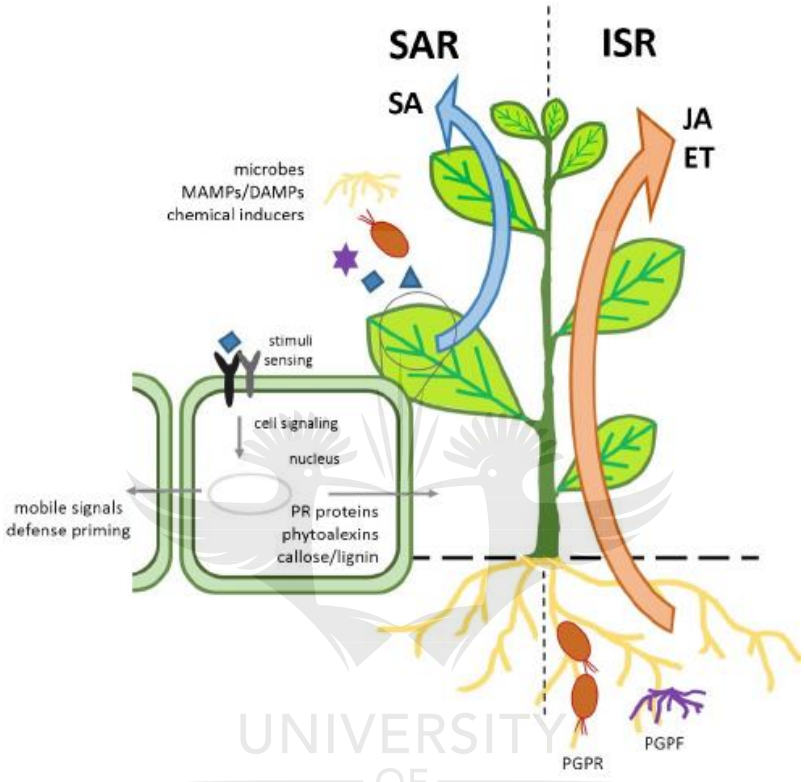

Figure 2.4: The two forms of systemic resistance in plants, SAR and ISR. These two forms of resistance have demonstrated to be effective against a broad spectrum of pathogens. Upon detection of extracellular stimuli such pathogen attack *via* M/PAMPS or chemical inducers, activation of signalling cascades occurs, resulting in the expression of defence-related genes (phytoalexin production and synthesis of PR-proteins) and cell wall reinforcement associated with systemic acquired resistance (SAR, left). SAR is mainly mediated by salicylic acid (SA), with the phytohormone spreading to distal parts of the plant activating SAR. On the other hand, induced systemic resistance (ISR, right) results from colonisation of the plant roots by beneficial soil microbes such as plant growth-promoting rhizobacteria (PGPR) and symbiotic fungi (PGPF) leading to the protection of above-ground plant tissues against pathogen infection. The main phytohormones mediating this form of resistance are jasmonic acid (JA) and ethylene (ET) (taken from Burketova *et al.*, 2015).

These two forms of resistance, thus, demonstrate that phytohormones play important roles in plant defence response against various biotic and abiotic stresses and, in addition to that, they are vital to growth and developmental processes. Phytohormones are compounds synthesised in low concentrations that regulate cellular processes in plants. They play a crucial role as chemical messengers, coordinators for signal transduction in pathways as well as plant mediators for defence response against stresses (Fujita *et al.*, 2006; Ingle *et al.*, 2006; Pieterse

et al., 2009; Andolfo & Ercolano, 2015; Bigeard *et al.*, 2015; Wani *et al.*, 2016). These various plant hormones include abscisic acid (ABA), indole-3 acetic acid/ auxin (IAA), cytokinins (CKs), brassinosteriods (BRs), gibberellins (GAs), strigolactones and peptide hormones (Bari & jones, 2009). Amongst the plant hormones, SA, JA and ET together with ROS signalling pathways, have been shown to be mostly involved in the induced resistance pathways (SAR and ISR) in plant innate immunity (Rojo *et al.*, 2003; Fujita *et al.*, 2006; Andolfo & Ercolano, 2015). As mentioned previously, SA signalling mostly occurs upon biotrophic infection and also leads to SAR, whereas ET and JA signalling mostly occurs upon necrotrophic infection and also leads to ISR (Schreiber & Desveaux, 2008; Bari & Jones, 2009; Návarová *et al.*, 2012; Andolfo & Ercolano, 2015; Bigeard *et al.*, 2015).

As highlighted in **section 2.3.1**, the perception of pathogen presence by plants necessitates the recognition of conserved molecular features on pathogens, resulting in activation of lines of defence responses. Lipopolysaccharide (LPS) (Sanabria *et al.*, 2008; Madala *et al.*, 2011; Finnegan *et al.*, 2016; Ranf, 2016), epitopes from flagellin, elongation factor Tu and cold-shock protein from bacterial pathogens (Ingle *et al.*, 2006; Cheynier *et al.*, 2013), and ergosterol, β-glucans and chitin from fungal pathogens, are amongst the known M/PAMPs that trigger defence responses in plants (Zhao *et al.*, 2005; Zeidler *et al.*, 2010; Tugizimana *et al.*, 2012; Klemptner *et al.*, 2014; Mpofu & McLaren, 2014; Mhlongo *et al.*, 2016). The current study involves infection of sorghum by a bacterial pathogen (*B. andropogonis*) of which LPS from the pathogen is a potentially perceived M/PAMP by the host. The following section will provide general information regarding LPS as a MAMP.

2.3.3. Lipopolysaccharides as M/PAMPs in the perception of bacterial invasion

LPS, an amphiphilic glycolipid or lipoglycan is found in Gram-negative bacteria and some cyanobacteria, and is important for the environmental survival of these bacterial pathogens. LPS contributes towards the resistance of Gram-negative bacteria to antimicrobial compounds and challenging environments, with lipid A and inner core backbone rigidity contributing to resistance. This conserved feature is thermostable and is located on the outermost membrane of the Gram-negative bacteria (Newman *et al.*, 2000, 2002; De Castro *et al.*, 2010; Madala *et al.*, 2011; Ernst *et al.*, 2014; Di Lorenzo *et al.*, 2015; Zipfel, 2015; Mhlongo *et al.*, 2016; Ranf, 2016; Choma *et al.*, 2017). However, its structure may differ within and across bacterial species

to an extent that a bacterial cell may contain varying LPS forms. Under different environmental conditions, varying LPS structures arise, thus contributing to the diversity in LPS structures (Di Lorenzo *et al.*, 2015; Ranf, 2016; Choma *et al.*, 2017).

There are three main structural components of LPS, namely the O-chain/antigen, core oligosaccharide and the lipid A, as shown in **Figure 2.5**. These three components are linked *via* covalent bonds. Stabilisation of the LPS structure is due to attraction between negative charges on the lipid A domain and core oligosaccharide, and cations such as $Ca^{2+}$and Mg^{2+} (Newman *et al.*, 2000; De Castro *et al.*, 2010; Madala *et al.*, 2011; Ranf *et al.*, 2015; Zipfel, 2015; Ranf, 2016; Choma *et al.*, 2017). In terms of bacterial colony appearance, the absence of the O-chain/O-antigen results in rough LPS and the presence of the same component results in smooth LPS (De Castro *et al.*, 2010; Madala *et al.*, 2011; Di Lorenzo *et al.*, 2015; Ranf, 2016).

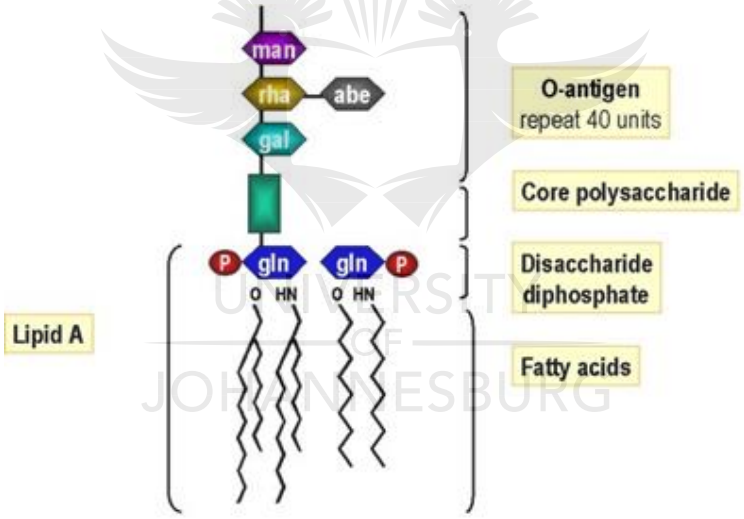

Figure 2.5: A general LPS structure. The macromolecule is made up of three structural components; lipid A, the innermost component linked to the outer membrane; a core oligosaccharide located in the mid-section and connected to the lipid A component *via* covalent bonds, and an O chain forming the outermost component exposed to the environment (taken from Acharya, 2013).

The O-chain, also known as O-polysaccharide or O-antigen is the hydrophilic outermost component of LPS exposed to the environment (Madala *et al.*, 2011). This component differs with species of bacteria as well as the strain (structural diversity), and takes part in the protection of the bacteria in challenging surroundings (Di Lorenzo *et al.*, 2015; Ranf, 2016). On the other hand, the core oligosaccharide in LPS is the component located between the O-antigen and lipid A, joining these two structural components together. This component is divided into an inner core and outer core. The conserved inner core is the section that is directly

connected to the lipid A domain *via* 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) (Newman *et al.*, 2000; Di Lorenzo *et al.*, 2015; Ranf, 2016). Additionally, the lipid A domain is a hydrophobic di-glucosamine that is linked to the bacterial outer membrane (OM) (Sanabria & Dubery, 2006; De Castro *et al.*, 2010; Sanabria *et al.*, 2010; Ranf, 2016). This domain is highly conserved and contributes to LPS stability. Although lipid A is highly conserved, certain factors such as environment and growth conditions may lead to differences in its structure, thus affecting its biological activity (Di Lorenzo *et al.*, 2015). Lipid A is perceived as a M/PAMP by some host plants (Zeidler *et al.*, 2010). Recently a plant LPS receptor was identified in *Arabidopsis thaliana*, a member of the cruciferous plants (Brassicaceae family). This LipoOligosaccharide-specific Reduced Elicitation (LORE) receptor is reported to sense the lipid A domain of LPS. However, LPS sensing by this receptor only occurs for LPS from *Pseudomonas* and *Xanthomonas* bacterial species. Thus far no other LPS receptor has been identified in other plant families (Zipfel, 2015; Ranf, 2016).

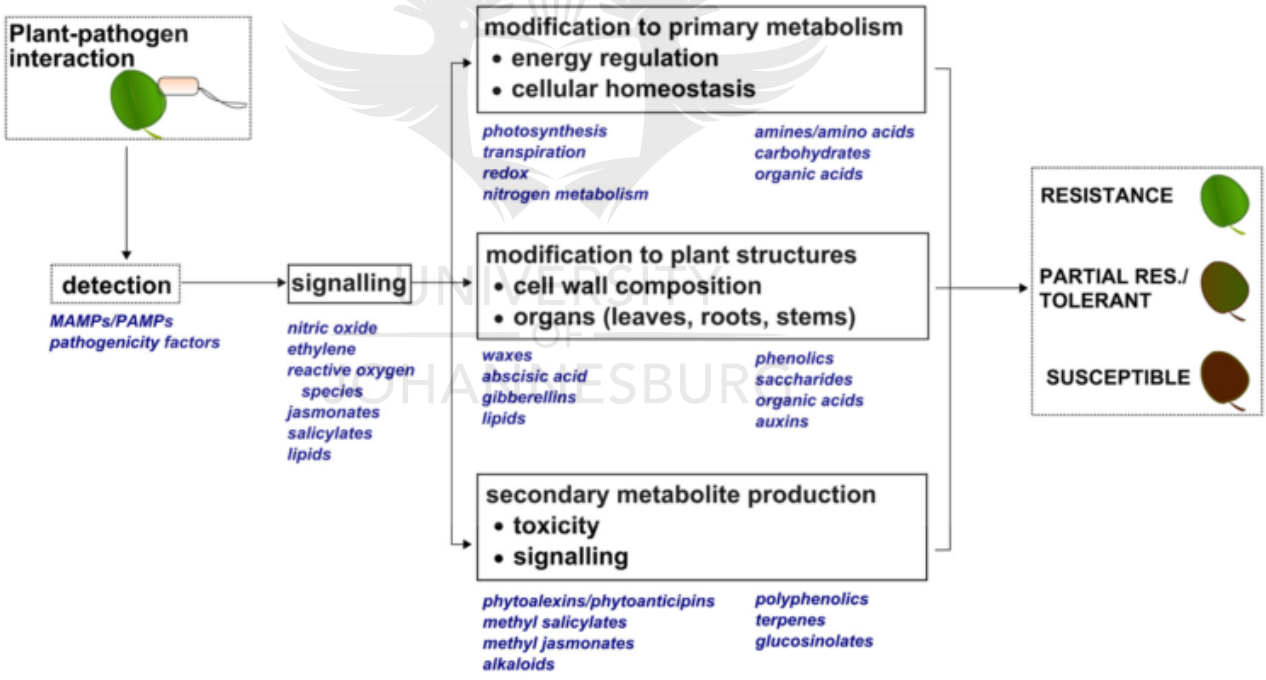

Figure 2.6: A summary of some molecular and physiological events triggered upon plant–pathogen interactions. The detection of plant pathogen *via* conserved molecular features on the pathogen, triggers the activation of a chain of signalling cascades mediated by signalling molecules such as SA, JA, MeJA and some components of the plant lipidome. These signalling events result in metabolic reprogramming in primary and secondary metabolism and immune responses such as cell wall reinforcement/strengthening (physical defence) and defence-related secondary metabolite production (chemical defence). The outcome of these immune responses ultimately defines whether the plant phenotype is either susceptible, partially resistant/tolerant, or resistant (taken from Heuberger *et al.*, 2014).

In summary, the molecular and physiological events possibly occurring as a result of the interaction between a plant and a pathogen are outlined in **Figure 2.6**. The outcome of these plant defence response is determined by the plant's ability to detect the pathogen presence *via* M/PAMPs such as LPS, activating defence responses and the pathogen's ability to suppress the immune system of the plant (Pieterse *et al.*, 2009; Pritchard & Birch, 2011). The recognition of such stimuli by plants activates signalling cascades resulting in profound fluctuations in the plant's metabolism (primary and secondary) (Anjum *et al.*, 2013; Balmer *et al.*, 2013). These fluctuations include defence-related secondary metabolites production – a key component of the plant defence repertoire, which are of particular interest in this study (Ahuja *et al.*, 2012; Balmer *et al.*, 2013).

2.3.4. Plant metabolism – focus on defence-related secondary metabolism

Metabolism entails the summation of various biochemical reactions within a living organism (van der Fits & Memelink, 2000; Vince & Zoltán, 2011). The chemical entities, products or intermediates of these reactions are called metabolites (Devika & Koilpillai, 2012) and classified as either primary or secondary, and sharing central metabolic pathways (van der Fits & Memelink, 2000; Devika & Koilpillai, 2012). Plant primary metabolites are generally known to directly participate in the plant's functioning, including growth, storage, reproduction and development processes (Bourgaud *et al.*, 2001; Hong *et al.*, 2016; Tenenboim & Brotman, 2016). These compounds also play a role of providing energy during defence responses against pathogens (Andolfo & Ercolano, 2015). Nucleic acids, lipids, carbohydrates and proteins are examples of such metabolites (Vince & Zoltán, 2011). With regard to chemical structure and profusion, primary metabolites are conserved to a greater degree than secondary metabolites (Hong *et al.*, 2016). Secondary metabolites conversely, are compounds that might have no direct influence in plant growth and development but are still required for plant survival, well-being and interactions with the environment (van der Fits & Memelink, 2000; Bourgaud *et al.*, 2001; Cheynier *et al.*, 2013; Piasecka *et al.*, 2015; Hong *et al.*, 2016). Together primary and secondary metabolites make up the plant's metabolome. Although much is known about plant metabolites, the work on identification and characterisation of the whole plant metabolome (estimated to be more than 200,000 metabolites) is still far from being done (Kliebenstein, 2012; Hong *et al.*, 2016).

Plants biosynthesise an immense quantity of various secondary metabolites. Functions of these include (i) plant defence responses against pathogen attack – through mechanical barriers formation, hindering pathogen invasion or killing of pathogens with their antiviral, antifungal and antibiotic properties, (ii) deterring feeding herbivores, (iii) luring of pollinators and seed dispersal agents, (iv) UV damage protection and (v) contribution to fruit taste and colour (van der Fits & Memelink, 2000; Awika & Rooney, 2004; Bino *et al.*, 2004; Cheynier *et al.*, 2013; Piasecka *et al.*, 2015; Sun *et al.*, 2016; Tenenboim & Brotman, 2016). Additionally, secondary metabolites are involved in adjustment of plants to their environment as well as the interactions between these two systems (Buchanan *et al*. 2000; Piasecka *et al.*, 2015; Sun *et al.*, 2016).

Secondary metabolites have diversified chemical structures and profusion compared to primary metabolites, with noticeable variations in the plant kingdom. The tissue or family of the plant influences the type of secondary metabolites synthesised – which contributes to the diversity of secondary metabolites (Vince & Zoltán, 2011; Piasecka *et al.*, 2015; Hong *et al.*, 2016). Plant secondary metabolite benefits/uses are not confined to plants. These natural plant products are now being widely used in various industries such as the agricultural sector, in insecticide manufacturing, pharmaceutical industry, cosmetics industry, fragrance industry and are gaining popularity in the nutraceutical sector (van der Fits & Memelink, 2000; Bourgaud *et al.*, 2001; Devika & Koilpillai, 2012). Classification of secondary metabolites can be according to their chemical structure, solubility but mostly metabolic pathways they derive from (Bourgaud *et al.*, 2001; Devika & Koilpillai, 2012; Sun *et al.*, 2016). Terpenes, phenolic compounds and nitrogen/sulphur-containing compounds are generally regarded as the three main categories of plant secondary metabolites (**Figure 2.7**). Of these, terpenes are the largest group whereas phenolic componds are the most wide-ranging across the plant kingdoms (Bourgaud *et al.*, 2001; Rohdich *et al.*, 2002; Cheynier *et al.*, 2013; Sun *et al.*, 2016).

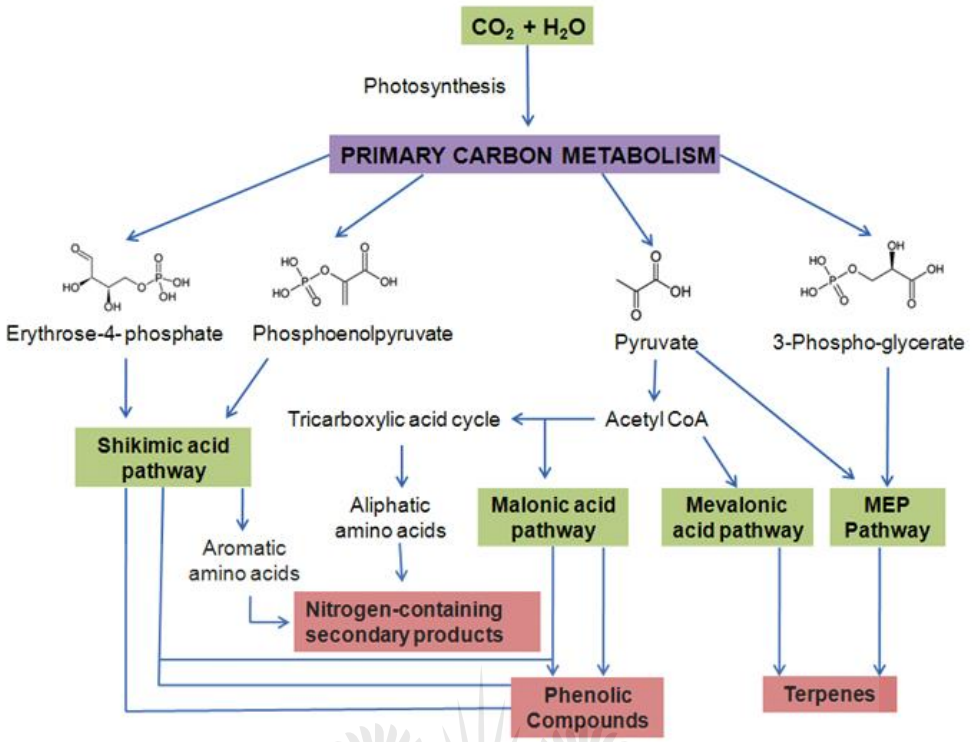

Figure 2.7: Simplified illustration of the links between primary and secondary metabolism and the major pathways leading to biosynthesis of secondary metabolites. Primary and secondary metabolites share principal metabolic pathways and biosynthesis of secondary metabolites requires plants to take into account primary metabolic pathways. The three classes of secondary metabolites, which are phenolic compounds, terpenes and nitrogen containing compound, are also indicated. **Abbreviation**: MEP = methylerythritol phosphate pathway (non-mevalonate pathway) (adapted from http://nptel.ac.in/courses/102103016/module4/lec31/2.html, accessed 04/04/2017).

2.3.4.1. Secondary metabolites in sorghum

The major group of secondary metabolites normally found in sorghum is phenolic compounds, which can be divided into two main groups, being phenolic acids (mostly derived from cinnamic acid or benzoic acid) and flavonoids. Generally, phenolic acids are conjugated with organic acids or sugars. The bran of sorghum grain is regarded as the compartment that contains high levels of phenolic acids. The composition together with the relative concentrations of phenolic compounds differ in sorghum cultivars, and factors contributing thereto include genetics, age of the plant and the environment in which the plant is grown (Woodhead, 1981; Awika & Rooney, 2004;Taylor *et al.*, 2014; Kang *et al.*, 2016). In terms of phenolic compound content, it has been reported that sorghum ranks highest measured against cereals such as rye, wheat, barley and millet (Awika, 2011; Kang *et al.*, 2016). Amongst other defence-related secondary metabolites, sorghum flavonoids play a role against pathogen attack (Awika & Rooney, 2004). **Table 2.2** indicates some secondary metabolites that have been reported in sorghum (independent of function).

Table 2.2: Major classes of secondary metabolites that have been reported in sorghum, independent of their function.

Group	Class	Secondary metabolite	Source
Flavonoid	Flavan-4-ols	Apiferol, luteoferol	Awika & Rooney, 2004; Boddu *et al.*, 2004; Ibraheem *et al.*, 2010, 2015; Awika, 2011; Poloni & Schirawski, 2014
	Flavones	Luteolin, apigenin 7-O-methyl luteolin	Awika & Rooney, 2004; Du *et al.*, 2010; Awika, 2011; Poloni & Schirawski, 2014; Kang *et al.*, 2016
	Flavanones	Naringenin, eriodictyol, eriodictyol 5-O-β-glucoside	Awika & Rooney, 2004; Ibraheem *et al.*, 2010, 2015; Awika, 2011; Kang *et al.*, 2016
	Flavan-3-ols	Catechin, epicatechin 7-O-Methyl catechin, 7-O-Methyl afzelechin	Awika, 2011; Kang *et al.*, 2016
	Flavonol	Kaempferol, quercetin	Awika, 2011; Poloni & Schirawski, 2014; Kang *et al.*, 2016
	Proanthocyanidins/ condensed tannins	Polyflavan-3-ol, procyanidin, proluteolinidin, proapigeninidin, prodelphinidin	Awika & Rooney, 2004; Awika, 2011
	Dihydroflavonol	Taxifolin, taxifolin 7-O-β-glucoside	Awika & Rooney, 2004; Kang *et al.*, 2016
	Anthocyanidins	Apigeninidin, luteolinidin, 5-methoxyluteolinidin, 7-methoxyapigeninidin, 7-O-methylapigeninidin, fisetinidin, cyaniding, pelargonidin, caffeic acid ester of arabinosyl 5-O-apigeninidin	Awika & Rooney, 2004; Boddu *et al.*, 2004; Wu & Prior, 2005; Ibraheem *et al.*, 2010, 2015; Awika, 2011; Poloni & Schirawski, 2014
Phenolamides		N1,N8-Caffeoyl-feruloyl spermidine, N1,N8-Dicaffeoyl spermidine	Kang *et al.*, 2016
Phenolic acids		Syringic acid, protocatechuic acid, p-coumaric acid, sinapic acid, ferulic acid, caffeic acid, o-coumaric acid, salicylic acid, p-hydroxybenzoic acid, gallic acid, gentisic acid	Awika & Rooney, 2004; Kang *et al.*, 2016

2.3.4.2. Phytoanticipins and phytoalexins, the major players in plant chemical defences

As mentioned above, the intricate defence system used by plants entails the production of a wide range of various defence-related metabolites of which the contribution to plant innate immunity is crucial. These compounds fall under three major categories namely, isoprenoids, alkaloids and shikimates. Modifications of compounds belonging to these three major classes

results in a broad spectrum of compounds with diverse biological functions (Morrissey & Osbourn, 1999; Dewick, 2002; Kliebenstein, 2012; Balmer *et al.*, 2013; Cheynier *et al.*, 2013; Sun *et al.*, 2016). The role of defence-related secondary metabolites in plant innate immunity involves the protection of plants from pathogen attack through antimicrobial activity. In a natural environment, production of these antimicrobial compounds contibutes to the limitation of total host plant takeover by pathogens, where activation or biosynthesis is stimulated by the detection of conserved features (the M/PAMPs) on the pathogen (**section 2.3.1**) (Bourgaud *et al.*, 2001; Liu *et al.*, 2010; Kliebenstein, 2012; Tugizimana *et al.*, 2012; Cheynier *et al.*, 2013; Piasecka *et al.*, 2015).

Defence-related secondary metabolites have various classifications, but mostly are categorised according the mode of biosynthesis, regulation and biological activity, into phytoanticipins or phytoalexins. Two definitions have been portrayed for the former. Phytoanticipins are defined as defence-related compounds that already exist in the plant (preformed) even before pathogen invasion or compounds that exist as inactivated precursors in a healthy plant but, upon pathogen invasion, are activated to perform their antimicrobial functions. The activation of these (constitutively present) compounds is carried out by particular enzymes present in the plant, and pathogen invasion can result in elevated levels of phytoanticipins (Osbourn, 1996; Morrissey & Osbourn, 1999; Meyer *et al.*, 2015; Piasecka *et al.*, 2015; Cuperlovic-Culf *et al.*, 2016; Pastorczyk & Bednarek, 2016). Phytoalexins, on the other hand, are generally defined as defence-related compounds with antimicrobial activity produced *de novo* upon- or post-pathogen invasion (induced). There has been developments on the term phytoalexin with this not only referring to defence compounds induced by biotic stressors, but also to defence compounds produced with the introduction of abiotic stressors (Dixon, 1999; Tugizimana *et al.*, 2012; Balmer *et al.*, 2013; Cheynier *et al.*, 2013; Klemptner *et al.*, 2014; Piasecka *et al.*, 2015; Finnegan *et al.*, 2016).

Phytoanticipins include defence metabolites of the saponins, glucosinolates and cyanogenic glycoside chemical classes, which have been thoroughly studied (Morrissey & Osbourn, 1999; Balmer *et al.*, 2013; Piasecka *et al.*, 2015). Saponins result from glycosylation of triterpenoid or steroid. Some examples hereof are the triterpenoids avenacin A-1, avenacin A-2, avenacin B-1 and avenacin B-2 that have been reported in oat roots responding to fungal attack. Other examples are the steroidal glycoalkaloids α-chaconine in potato and α-tomatine in tomato (Morrissey & Osbourn, 1999; Piasecka *et al.*, 2015). The class of cyanogenic glycosides, on

the other hand, comprises products of the reaction of amino acids to oximes followed by glycosylation, which are stored mostly in an inactive form. When the host plant is posed with a threat, cyanogenic glycosides are degraded by enzymes, producing poisonous hydrogen cyanide in defence against biotic stress in a process termed cyanogenesis. In the same reaction, a ketone or aldehyde is produced together with the cyanide. The amino acid precursors in the synthesis of cyanogenic glycosides are primary metabolites and include valine, phenylalanine, leucine, isoleucine and tyrosine (Poulton, 1990; Morrissey & Osbourn, 1999; Winkel, 2004; Piasecka *et al.*, 2015).

Sorghum, lima beans and cassava are cyanogenic plants known to produce cyanogenic glycosides (Poulton, 1990; Winkel, 2004). In sorghum, the cyanogenic glycoside dhurrin forms part of preformed defence compounds (Poulton, 1990; Dicko *et al.*, 2006; Madala *et al.*, 2014). However, this metabolite is considered more of an insect feeding deterrent than a defence against microbial pathogen attack, since in a healthy plant tissue dhurrin is constitutively present in elevated levels and attack by some pathogens does not seem to alter its levels (Nicholson *et al.*, 1987; Dicko *et al.*, 2006; Du *et al.*, 2010; Liu *et al.*, 2010; Mizuno *et al.*, 2014; Piasecka *et al.*, 2015). Although not found in sorghum, another related class of phytoanticipins comprises the glucosinolates. The latter are glucosides derived from amino acid precursors similar to cyanogenic glycosides, but contain sulphur. It has been suggested that the pathway that synthesises glucosinolates branched off from the cyanogenic glycoside synthesis pathway (Morrissey & Osbourn, 1999; Piasecka *et al.*, 2015).

As highlighted above, in addition to the pre-formed defence-related metabolites (phytoanticipins), the plant immune responses involve *de novo* biosynthesis of antimicrobial compounds known as phytoalexins. The latter have an important role in the plant defence repertoire, acting on a range of pathogens (Ahuja *et al.*, 2012; Balmer *et al.*, 2013). An array of these induced defence compounds, reported in plants, include those originating from phenylalanine and alkaloid derivatives of the amino acid tryptophan (Meyer *et al.*, 2015). Production of phytoalexins is regulated through plant hormones. Phytoalexins mostly accumulate or are concentrated at the site of infection as well as regions around the infection site with their production limiting the spread of pathogen infection. The rate at which these compounds are produced is critical in plant defence (Nicholson *et al.*, 1987; Snyder & Nicholson, 1990; Hipskind *et al.*, 1990). Presence/production of phytoalexins serves as an indication of disease resistance in a host plant as they are molecular markers thereof (Ahuja *et*

al., 2012; Poloni & Schirawski, 2014). The type of phytoalexin produced in plants are to some extent restricted to a plant species or plant family (some phytoalexins are only found in certain plant families) (Bennett & Wallsgrove, 1994; Morrissey & Osbourn, 1999; Ahuja *et al.*, 2012; Jeet *et al.*, 2014). Examples of phytoalexins derived from phenylalanine are pisatin in pea (Zhao *et al.*, 2005; Métraux *et al.*, 2009), 3-deoxyanthocyanidins in sorghum (Poloni & Schirawski, 2014) and sakuranetin in rice (Piasecka *et al.*, 2015). Synthesis of terpenoid phytoalexins in response to pathogen challenge has also been reported, these include 6-methoxygossypol and hemigossypol in cotton (Bennett & Wallsgrove, 1994), momilactones in rice (Balmer *et al.*, 2013; Jeet *et al.*, 2014), zealexins in maize (Jeet *et al.*, 2014) and capsidiol and solavetivone in tobacco (Tugizimana *et al.*, 2012). Camalexin, an indole alkaloid, has been reported in *Arabidopsis* (Brassicaceae family) (Ahuja *et al.*, 2012; Finnegan *et al.*, 2016).

Dating back to the 1980s a class of phytoalexins, not previously known in sorghum in response to pathogen attack, was identified: the 3-deoxyanthocyanidins (**Figure 2.8**). The latter which are anthocyanidins not frequently occurring, were detected in sorghum (as *de novo* synthesised) upon challenge by a fungal pathogen *Colletotrichum graminicola*, and were found to contribute to resistance (Nicholson *et al.*, 1987; Snyder & Nicholson, 1990; Poloni & Schirawski, 2014). Nicholson and colleagues (1987) demonstrated that challenge of sorghum by a non-pathogenic fungus, *Helminthosporium maydis*, also resulted in accumulation of this class of compounds. Apigeninidin and luteolinidin were found to be the two main, dominant 3-deoxyanthocyanidin phytoalexins, rapidly accumulating in sorghum post-infection. Under pathogen challenge, the sorghum epidermal cells developed inclusion structures (harbouring these 3-deoxyanthocyanidin phytoalexins), which then migrated to the location of infection – maturing and becoming more pigmented (orange-red-brown, primarily as a result of apigeninidin and luteolinidin presence) in the process and finally releasing the phytoalexins to limit the infection (Nicholson *et al.*, 1987; Hipskind *et al.*,1990; Snyder & Nicholson, 1990; Snyder *et al.*, 1991; Morrissey & Osbourn, 1999; Boddu *et al.*, 2004; Basavaraju *et al.*, 2009).

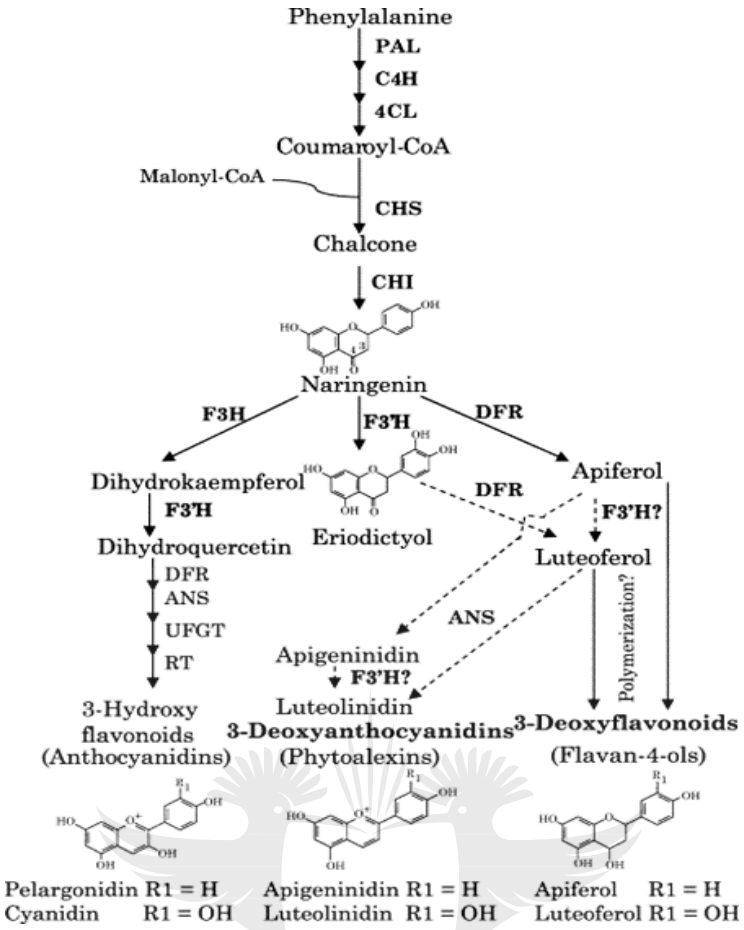

Figure 2.8: Biosynthetic pathway leading to the synthesis of 3-deoxyanthocyanidin phytoalexins and other defence-related secondary metabolites in sorghum. The 3-deoxyanthocyanidins are a class of flavonoid compounds, part of phenolic secondary metabolites. They share naringenin as a precursor (and branching point) with 3-hydroxyflavonoids and 3-deoxyflavanoids. 3-deoxyanthocyanidins synthesis in light-independent. The introduction of DFR, ANS and F3′ H channels the pathway to the synthesis of 3-deoxyanthocyanidins. **Abbreviations**: PAL= phenylalanine ammonia lyase, C4H= cinnamate 4-hydroxylase, 4CL= coumarate 4-ligase, CHS= chalcone synthase, CHI= chalcone isomerase, DFR=dihydroflavonol reductase, ANS= anthocyanidin synthase and F3′ H= flavonoid 3′ hydroxylase (taken from Boddu *et al.*, 2004).

The caffeic acid ester of arabinosyl 5-O-apigeninidin, 5-methoxyluteolinidin and 7-methoxyapigeninidin are derivatives of 3-deoxyanthocyanidin phytoalexins, also reported in sorghum (Boddu *et al.*, 2004; Wu & Prior, 2005; Ibraheem *et al.*, 2010; Poloni & Schirawski, 2014). The phytohormone MeJA has been reported to trigger accumulation of 3-deoxyanthocyanidins (Liu *et al.*, 2010). In addition to 3-deoxyanthocyanidin phytoalexins, luteolin and apigenin, flavone phytoalexins, have also identified in sorghum as defence- related (Du *et al.*, 2010; Ahuja *et al.*, 2012; Jeet *et al.*, 2014; Poloni & Schirawski, 2014). The differences between these flavones and the 3-deoxyanthocyanidins – luteolinidin and apigeninidin – arise from their mode of synthesis. Conversion of naringenin or eriodictyol (flavanones) by the enzyme flavone synthase II (FNSII) leads to apigenin and luteolin (flavones) synthesis, respectively. In contrast the conversion of naringenin or eriodictyol to

apiferol or luteoforol (flavan-4-ols), respectively, by flavanone 4-reductase (FNR) leads to 3-deoxyanthocyanidins synthesis (**Figure 2.8**). Luteolin and luteolinidin differ from apigenin and apigeninidin in that their synthesis involves hydroxylation of the B-ring of naringenin on position 3′ by flavonoid 3′-hydroxylase (F3′H), which does not occur in the synthesis of the latter (Ayabe *et al.*, 2010; Du *et al.*, 2010; Mizuno *et al.*, 2016). With much known about involvement of phytoalexins in defence responses, the molecular mechanisms by which these phytoalexins act against microbial pathogens is, however, not fully understood.

With regard to bacterial pathogens, the 3-deoxyanthocyanidin, apigeninidin was reported to limit bacterial growth on agar plates in an investigation carried out by Stonecipher *et al.*, (1993). The phytoalexin was shown to have antimicrobial activity against Gram-negative bacteria *Serratia marcescens*, *Escherichia coli* and *Shigella flexneri*, and Gram-positive bacteria *Streptococcus faecalis*, *Staphylococcus aureus*, *Bacillus cereus* and *Staphylococcus epidermidis* (Stonecipher *et al.*, 1993; Poloni & Schirawski, 2014). However, in plants, there are still gaps with regards to metabolic changes and studies, on sorghum–bacterial interactions.

Although there has been a considerable number of studies focusing on plant immunity and a thorough exploration on certain facets thereof that has provided information enabling the understanding of underlying biochemical and molecular biological processes, there are still gaps in the field of plant–pathogen interactions leading to continuous research studies in the field (Berger *et al.*, 2007; Zipfel, 2009; Segonzac & Zipfel, 2011; Martinez-Medina *et al.*, 2016; Tenenboim & Brotman, 2016; Xin *et al.*, 2016). Thus, the work presented in this dissertation, is an untargeted liquid chromatography-mass spectrometry (LC-MS)-based metabolomics approach to comprehensively investigate the biochemistry of the induced defensive state in *S. bicolor* in response to *B. andropogonis* infection.

2.4. Metabolomics as a tool to investigate inducible plant defence responses

Metabolomics has demonstrated to be an essential functional genomics approach in the study of biological systems at various levels. The research field focuses on the comprehensive analysis of all metabolites (small molecules ≤ 1500 Da) collectively known as the metabolome – at the cell, tissue, organ and organism level under specific physiological conditions, representing a diverse array of metabolic pathways and intermediary metabolism (Goodacre

et al., 2004; Bino *et al.*, 2004; Hall, 2006; Allwood *et al.*, 2008; Johnson & Gonzalez, 2012; Milne *et al.*, 2013; Misra *et al.*, 2017). This recently emerged technology is the final of the logical flow of the 'omics' technologies (genomics→ transcriptomics→ proteomics→ metabolomics) and is complementary to these other approaches (Goodacre *et al.*, 2004; Richard & Louise, 2011). Metabolomics facilitates our understanding (functionally) of the cellular processes occurring in biological systems and the dynamics thereof. The metabolomics approach, in combination with other established technologies, is unremittingly advancing and continues to increase our knowledge on (i) underlying biochemical processes in complex biological systems and (ii) the functioning of organisms as integrated biological systems (Hall, 2006; Hall *et al.*, 2008; Vidal, 2009; McKnight, 2010; Ray, 2010; Nanda *et al.*, 2011; Tugizimana *et al.*, 2013; Sévin *et al.*, 2015; Jorge *et al.*, 2016; Misra *et al.*, 2017).

In addition to metabolomics, the other widely used functional genomics tools *i.e.* transcriptomics and proteomics, provide a broad genome coverage and are more fitting in giving insight in the different layers of regulation in systems biology (Mathew & Padmanaban, 2013; Tugizimana *et al.*, 2013; Fuhrer & Zamboni, 2015). However, these approaches do not always provide adequate information regarding protein function (ideally displayed by the biochemical phenotype) (Verpoorte *et al.*, 2008). On the contrary, the metabolome which is the downstream product of the flow from gene to function, is more sensitive to changes in metabolic fluxes and enzyme activity in comparison to the transcriptome or proteome. Moreover, the changes in the metabolome can be easily detected and monitored (Kell *et al.*, 2005; Richards *et al.*, 2010; Johnson & Gonzalez, 2012). The metabolomics approach thus, has emerged as an indispensable tool as it offers an actual 'snap-shot' in time of the physiological state and biological activities of an organism (with metabolites acting as direct phenotypic signatures) (Bino *et al.*, 2004; Guy *et al.*, 2008; Verpoorte *et al.*, 2008; Tugizimana *et al.*, 2013; Yin & Xu, 2014; Jorge *et al.*, 2016). Therefore, biological systems subjected to genetic modifications, pathological conditions and other stresses can be investigated using this approach. As such metabolomics is currently being used as an investigative tool in various biological research such as plant natural studies, drug discovery, diagnostics, understanding plant biochemistry and in nutraceutics (Nicholson *et al.*, 1999; Tugizimana *et al.*, 2013; Beisken *et al.*, 2015; Kell & Oliver, 2016). Although metabolomics has certain advantages over the other 'omics' technologies, an integration of the strategies gives a holistic understanding of the dynamics of biological systems (Bino *et al.*, 2004; Guy *et al.*, 2008; Yin & Xu, 2014; Jorge *et al.*, 2016).

The field of plant metabolomics has been of interest recently in the science community allowing more research to be carried out – partly due to its varied applications. This field targets studying of plant systems at molecular level – offering non-biased plant tissue metabolome characterisation in relation to environmental responses. Metabolic changes that occur in plants best reflect modifications in plant genome and interactions between plants and herbivores or pathogens and their environments (Hall, 2006; Allwood *et al.*, 2008; Hall *et al.*, 2008; Misra *et al.*, 2017). Recent advances of non-targeted multivariate tools, functional in the analysis of a wide array of plant metabolite profiles, has also supported the growing interest in plant metabolomics (Guy *et al.*, 2008).

The various plant studies employing metabolomics approach include (i) denoting alterations in metabolic fluxes, (ii) metabolic pathways analysis, (iii) providing a link between plant genotype and phenotype (Fiehn *et al.*, 2000; Weckwerth & Fiehn, 2002; Sumner *et al.*, 2003; Bhalla *et al.*, 2005; Hall, 2006; Kim *et al.*, 2011), (iv) characterisation of 'silent plant phenotypes'(Weckwerth *et al.*, 2004; Hall, 2006), (v) screening for plants with medicinal properties (Allwood *et al.*, 2008), (vi) examining significant modifications caused by random genetic mutation (Hall, 2006), (vii) screening populations of induced or spontaneous mutations (Hall, 2006; Allwood *et al.*, 2008), (viii) plant development strategies in breeding programmes (Hall, 2006; Harrigan *et al.*, 2007) and (ix) the study of plants under both biotic and abiotic stress – to understand changes that occur during stress, denoted by the plant's metabolic alterations. There are still gaps in understanding the mechanisms by which plants respond to biotic stress and metabolomics is being employed to shed more light regarding these mechanisms, especially on how plants defend themselves against pathogen attack – given that the nature of interaction varies with microbial agents and plant species (Bhalla *et al.*, 2005; Guy *et al.*, 2008; López-Gresa *et al.*, 2010; Tenenboim & Brotman, 2016).

Metabolomics studies generally fall into three categories, *i.e.* targeted, semi-targeted and non-targeted. Briefly described, targeted metabolomics studies focus on the quantification of a specific group of metabolites and involves the use of standards to track the changes of the metabolites of interest being quantified. Semi-targeted analysis falls between targeted and non-targeted approaches and similar to the former, the identities of the metabolites are known prior to data acquisition. On the contrary, the non-targeted approach is a high-throughput analysis which takes into account all possible metabolites in a sample, hence quantification seems impractical. General steps involved in metabolomics studies include sample collection, sample

preparation, data acquisition, data processing, data analysis, compound identification and biological interpretation. A summary of this multistep workflow employed in metabolomics studies is illustrated in **Figure 2.9** (Xiao *et al.*, 2012; Dunn *et al.*, 2013; Putri *et al.*, 2013; Naz *et al.*, 2014; Johnson & Carlson, 2015).

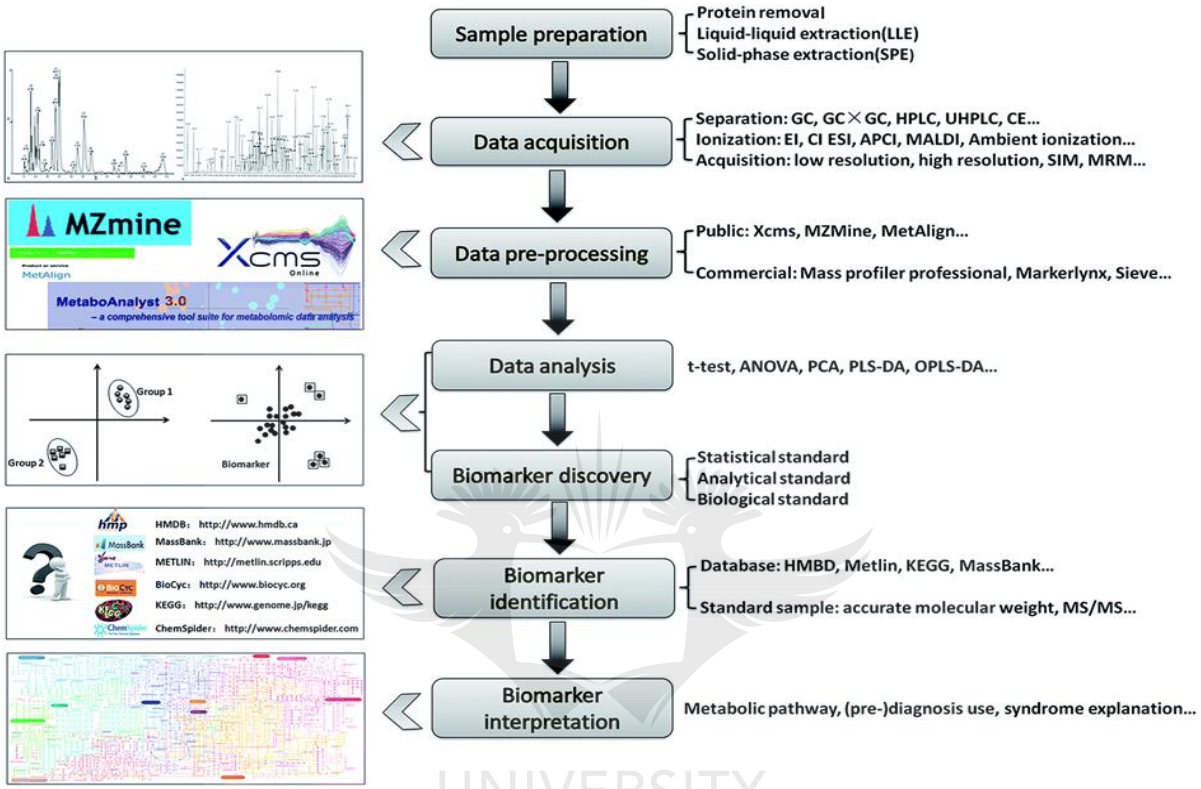

Figure 2.9: General steps involved in metabolomics studies. The first step involves the collection (harvesting and enzyme quenching) and preparation of samples (extraction, drying, reconstitution and/or derivatisation) for analysis. This is then followed by analysis of samples using various analytical platforms (*e.g.* LC-MS, GC-MS and NMR) to acquire data. The data mining/data analysis follows (data pre-processing and pre-treatment, univariate and multivariate analysis). Identification of metabolites present in the extracted samples and linking the results to the biological question (biological interpretation) conclude the steps in metabolomics studies (taken from Wang *et al.*, 2015).

Due to the wide array of compounds analysed in metabolomics studies with different chemical and physical characteristics and in varying concentrations, it is impossible for a single extraction protocol and analytical platform to cover the whole plant metabolome. Hence, it is important to determine the most suitable extraction procedure and analytical platforms for the study at hand. With regard to the latter, various platforms are being exploited in metabolomics studies and can be used in combinations to broaden the spectrum of metabolites to be covered (Allwood & Goodacre, 2010; Johnson & Gonzalez, 2012; Xiao *et al.*, 2012; Jorge *et al.*, 2016; Tenenboim & Brotman, 2016).

2.4.1. Liquid chromatography-mass spectrometry – a platform for plant secondary metabolite analysis

In order to determine the functions of particular metabolites in response to stimuli, examination of metabolic pathway fluxes and the study of the nature of the metabolome, – simultaneous metabolite identification and quantification is crucial. Multiple analytical platforms for metabolite identification are being used due to the vast heterogeneity of the plant metabolome which precludes a comprehensive metabolite analysis with a single standard technique. These platforms vary in their dynamic range, sensitivity, resolution, accuracy and eventually metabolite identification capabilities, and have advantages and disadvantages over each other (see **Table 2.3**) (Bino *et al.*, 2004; Allwood & Goodacre, 2010; Burgess *et al.*, 2011; Jorge *et al.*, 2016).

Liquid chromatography coupled to mass spectrometry (LC-MS) has become the most dominant analytical platform in metabolomics studies, mostly because of its high sensitivity and high resolution, enabling large-scale coverage of the metabolome (Putri *et al.*, 2013; Tugizimana *et al.*, 2017). The LC functions as the separation technique which is based on different compound elution rates – influenced by varying compound affinities for the mobile or stationary phase (*i.e.* differences in distribution coefficients). On the other hand, the MS functions as a detector, generating ions and measuring the mass-to-charge (*m/z*) ratios, giving information vital for compound structural elucidation (Bino *et al.*, 2004; Allwood *et al.*, 2008; Allwood & Goodacre, 2010; Balmer *et al.*, 2013; Tugizimana *et al.*, 2013; Gika *et al.*, 2014).

LC-MS has demonstrated to be a robust pre-eminent in analysing a wide array of metabolite classes with different physicochemical properties (*e.g.* polarity) compared to other analytical platforms. Metabolites, hydrophobic or hydrophilic in nature, can be separated and identified using LC-MS (Sumner *et al.*, 2003; Hall, 2006; Johnson & Gonzalez, 2012; Xiao *et al.*, 2012; Putri *et al.*, 2013; Gika *et al.*, 2014; Yin & Xu, 2014; Jorge *et al.*, 2016). Traditionally, most plant metabolomics studies make use of a non-polar reversed-phase (RP) stationary phase, in which silica is covalently bond to hydrophobic alkyl functional groups – mostly silica-C18 columns – together with polar aqueous mixture mobile phases such as water and methanol or water and acetonitrile (Allwood & Goodacre, 2010; Xiao *et al.*, 2012; Yin & Xu, 2014; Jorge *et al.*, 2016).

In most LC-MS-based metabolomics studies electrospray ionisation (ESI) is the common ionisation technique, regarded as a soft procedure that produces intact molecular ions with minimal metabolite fragmentation. This facilitates the determination of compound molecular weight and initial identification. ESI is frequently the preferred ion source when profiling unknown metabolites, as it allows acquiring of profiles in both positive and negative ionisation modes (Werner *et al.*, 2008; Xiao *et al.*, 2012; Gika *et al.*, 2014; Kind *et al.*, 2017). In the detection of metabolites in complex biological samples, LC-ESI-MS systems are increasingly becoming the preferred choice. The system allows more comprehensive coverage of the plant metabolome in relation to other currently used metabolite profiling systems (Xiao *et al.*, 2012; Milne *et al.*, 2013; Putri *et al.*, 2013; Yin & Xu, 2014; Wang *et al.*, 2015).

Over the years, improvements in LC-MS analytical instruments have enhanced extraction of more information from complex biological samples. Technological advances in LC-ESI-MS systems (mostly preferred in plant metabolomics studies) such as ultrahigh-performance liquid chromatographic (UHPLC) and introduction of advanced MS instrumentation, have led to improved peak resolution and increased sensitivity, robustness, detection specificity and functionality, respectively (Johnson & Gonzalez, 2012; Xiao *et al.*, 2012; Milne *et al.*, 2013; Putri *et al.*, 2013; Ernst *et al.*, 2014; Yin & Xu, 2014; Wang *et al.*, 2015). The sensitivity, reproducibility and reliability in quantitative analyses and high-resolution full scanning, lately provided by high-resolution, accurate-mass MS (HRMS), have also enhanced extraction of information regarding sample composition from diverse metabolite chemical classes. Ion trap-quadrupole-time-of-flight-MS (IT-Q-TOF-MS), quadrupole-time-of-flight-MS (Q-TOF-MS) and quadrupole-orbitrap-MS (Q-orbi-MS) are the presently available hybrid HRMS technologies being employed (Xiao *et al.*, 2012; Liu, 2012; Scigelova & Makarov, 2013; Glauser *et al.*, 2013; Vergeynst *et al.*, 2013; Yin & Xu, 2014; Ser *et al.*, 2015; Simader *et al.*, 2015; Rochat, 2016). Currently the LC-MS platform is being utilised to profile/investigate plant secondary metabolites in gene function, biomarker discovery, natural products elucidation, environmental perturbations and defence responses to mention but a few (Dixon, 2001; Sawada & Hirai, 2013; Tugizimana *et al.*, 2013; Cox *et al.*, 2014; Heuberger *et al.*, 2014; Hill & Roessner, 2015).

Table 2.3: Highlights of the advantages and disadvantages of some analytical platforms employed in metabolomics studies.

Technique	Advantage	Disadvantages	Source
Gas chromatography-mass spectrometry (GC-MS)	Good separation. High resolution. High sensitivity. High reproducibility. No ion suppression.	Not suitable for thermolabile compounds. Derivatisation required for non-volatiles. Extensive sample preparation. Destructive.	Khoo & Al-Rubeai, 2007; Allwood *et al.*, 2008; Balmer *et al.*, 2013; Tugizimana *et al.*, 2013; Wang *et al.*, 2015; Jorge *et al.*, 2016
Liquid chromatography-mass spectrometry (LC-MS)	No sample derivatisation required. Suitable for thermolabile compounds. Suitable for non-volatile compounds. Can analyse high molecular weight compounds. High throughput. High comprehensiveness. High mass accuracy. High sensitivity. High resolution.	Compound co-elution resulting ion suppression. Destructive. Extensive sample preparation.	Sumner *et al.*, 2003; Khoo & Al-Rubeai, 2007; Allwood & Goodacre, 2010; Tugizimana *et al.*, 2013; Jorge *et al.*, 2016
Nuclear magnetic resonance spectroscopy (NMR)	Non-destructive. Highly reproducible. Quantitative. Simple sample preparation. Solids and liquids compatible.	Poor sensitivity. Overlapping of signals. Increased sample amounts required.	Bino *et al.*, 2004; Allwood *et al.*, 2008; Allwood & Goodacre, 2010; Johnson & Gonzalez, 2012; Tugizimana *et al.*, 2013; Courant *et al.*, 2014; Wang *et al.*, 2015; Jorge *et al.*, 2016
Capillary electrophoresis-mass spectrometry (CE-MS)	Good detection limits. High resolution. Faster separation than LC. Separation of different charge-to-size ratios is possible. Detection of metabolites usually lost during derivatisation in GC-MS. Good separation for very polar compounds.	Technically demanding. Non-charged compounds separation is impossible. Poor reproducibility.	Sato *et al.*, 2004; Hall, 2006; Tugizimana *et al.*, 2013; Jorge *et al.*, 2016; Young & Alfaro, 2016

Liquid chromatography (LC)/NMR	Can analyse unstable metabolites. Good separation. Good for structural elucidation.	Low sensitivity. Low throughput. Requires solvent suppression.	Victoria & Elipe, 2003; Tugizimana *et al.*, 2013
Direct infusion mass spectrometry (DIMS)	Rapid metabolite fingerprinting. Increased sensitivity. High throughput.	Difficult to resolve isobars. Limited chemical information.	Allwood *et al.*, 2008; Allwood & Goodacre, 2010; Ernst *et al.*, 2014; Wang *et al.*, 2015
Fourier transform-infrared (FT/IR) spectroscopy	Non-destructive. Rapid sample analysis. High throughput. High reproducibility. Less sample preparation. No sample derivatisation required.	Intense IR water absorption. Structural information is limited.	Dunn & Ellis, 2005; Allwood *et al.*, 2008; Young & Alfaro, 2016
Raman spectroscopies	Rapid sample analysis. Minimised chemical bias. No water interferences. No sample derivatisation needed. Direct sample analysis.	Impossible for metabolite identification. Spectra are highly convoluted. Limited structural information.	Minai-Tehrani *et al.*, 2016; Young & Alfaro, 2016

Although LC-ESI-MS systems potentially offer comprehensive analysis of the metabolome, there are still gaps with regard to complete metabolome coverage. This is due to the limitations arising from issues such as the wide range of molecular weights of metabolites, varying metabolite concentrations and polarities, and matrix effects, such as ion suppression or enhancement as a result of co-elution (King *et al.*, 2000; Cech & Enke, 2002; Metz *et al.*, 2008; Theodoridis *et al.*, 2011; Van Der Hooft *et al.*, 2012; Putri *et al.*, 2013). However, there are continuous efforts as well as breakthroughs in addressing these limitations, together with technological advances of instruments and statistical modelling tools, to ensure a more comprehensive metabolome coverage (Likić *et al.*, 2010; Allwood *et al.*, 2011; Stahnke *et al.*, 2012; Ghosh *et al.*, 2012; Johnson *et al.*, 2015).

2.4.2. Nature of metabolomic data: statistical modelling in metabolomics

The generated metabolomics data (mostly in non-targeted approach) are high-dimensional, complex and information-rich data. Thus, data-handling and processing methodologies are crucial in order to extract information from such data sets (Ernst *et al.*, 2014; Misra & van der Hooft, 2016). Prior to statistical/chemometric analyses, processing of the complex raw data is normally performed. This data processing involves the critical steps of data pre-processing and pre-treatment. In LC-MS-based metabolomics studies, data pre-processing methods encompass baseline correction, peak detection, peak integration, peak alignment and peak annotation. On the other hand, data pre-treatment methods encompass normalisation, scaling, centering, transformation and dealing with missing values (Tugizimana *et al.*, 2013; Ernst *et al.*, 2014; Gika *et al.*, 2014; Yin & Xu, 2014; Alonso *et al.*, 2015; Misra & van der Hooft, 2016). Data processing makes use of automated tools (Tugizimana *et al.*, 2013) either open source (*e.g.* XCMS, MetaboAnalyst, etc.) or commercial (*e.g.* MarkerLynx^{TM}, Profiling solution, *etc.*) (Yin & Xu, 2014; Alonso *et al.*, 2015; Wang *et al.*, 2015; Misra & van der Hooft, 2016). The use and choice of these software packages varies with the nature of the data generated by an instrument (Tugizimana *et al.*, 2013; Gika *et al.*, 2014).

Following data processing (data cleaning), statistical analysis is performed, of which univariate and multivariate statistical analyses methods are employed in further extracting relevant information. These statistical methods reduce dimensionality of the data, explore the data, mine patterns in the data, and allow selection of statistically significant variables that are biologically relevant (Fiehn, 2002; van den Berg *et al.*, 2006, 2009; Jansen *et al.*, 2010; Kalogeropoulou,

2011; Stenlund, 2011; Misra & van der Hooft, 2016). The multivariate data analysis (MVDA) methods used include (i) unsupervised approaches, for example principal component analysis (PCA), simultaneous component analysis (SCA), canonical correlation analysis (CCA) and hierarchical cluster analysis (HCA) and (ii) supervised approaches, such as orthogonal partial least squares discriminant analysis (OPLS-DA), partial least squares discriminant analysis (PLS-DA), linear discriminant analysis (LDA), support vector machine (SVM) and genetic algorithms. A summary of these unsupervised and supervised MVDA methods is presented in **Table 2.4** (Yamamoto *et al.*, 2009; Priego-Capote *et al.*, 2012; Tugizimana *et al.*, 2013; Gika *et al.*, 2014; Yin & Xu, 2014; Misra & van der Hooft, 2016).

Table 2.4: A summary of some of the multivariate methods employed in metabolomics data analysis (adapted from Tugizimana *et al.*, 2013).

	Abbreviation	Term	Linearity /non-linearity assumption
Generally unsupervised models	CCA	Canonical Correlation Analysis	Linear
	CD-PCA	Clustering and Disjoint-Principal Component Analysis	Linear
	HCA	Hierarchical Clustering Analysis	Linear
	K-CCA	Kernel-Canonical Correlation Analysis	Non-linear
	K-PCA	Kernel-Principal Component Analysis	Non-linear
	KANN	Kohonen Artificial Neural Networks	Non-linear
	MSCA	Multi-level Simultaneous Component Analysis	Linear
	PCA	Principal Component Analysis	Linear
	SCA	Simultaneous Component Analysis	Linear
	W-PCA	Weighted-Principal Component Analysis	Linear
Generally supervised models	BANN	Back-prop Artificial Neural Networks	Non-linear
	ASCA	ANOVA-Simultaneous Component Analysis	Linear
	DA	Discriminant Analysis	Linear
	K-OPLS-DA	Kernel-Orthogonal Partial Least Squares-DA	Non-linear
	K-PLS-DA	Kernel-Partial Least Squares-DA	Non-linear
	N-PLS-DA	N-way PLS-DA	Linear
	OPLS-DA	Orthogonal Partial Least Squares-DA	Linear
	O2PLS-DA	Bidirectional Orthogonal PLS-DA	Linear
	OSC	Orthogonal Signal Correction	Linear
	PCDA	Principal Component Discriminant Analysis	Linear
	PLS	Partial Least Squares	Linear
	SIMCA	Soft Independent Modelling of Class Analogy	Linear
	PLS-DA	Partial Least Square Discriminant Analysis	Linear

In this study, of the unsupervised and supervised multivariate methods used in data analysis shown in **Table 2.4**, much focus will be on PCA and HCA, and OPLS-DA respectively. PCA is an unsupervised linear additive model, employed in metabolomics studies as the initial stage in data analysis – for data dimensionality reduction. This statistical method entails the linear transformation of a set of observations into linear orthogonal variables termed principal components (PCs) – projected to a low dimensional plot. The model facilitates the identification of patterns and trends in the data (Katajamaa & Orešič, 2007; Khoo & Al-Rubeai, 2007; Priego-Capote *et al.*, 2012; Balmer *et al.*, 2013; Putri *et al.*, 2013; Tugizimana *et al.*, 2013; Madala *et al.*, 2014; Alonso *et al.*, 2015). PCA scores plots visually shows sample clustering *i.e.* between and within samples' similarities and dissimilarities. The first principal component (PC1) explains most of the variance and the following PCs project the remaining variance – with each of the PCs constituting to the total variation (Steinfath *et al.*, 2008; Grata *et al.*, 2008; Werth *et al.*, 2010; Liland, 2011; Madala *et al.*, 2014; Naz *et al.*, 2014; Mhlongo *et al.*, 2016).

HCA is a clustering and visualisation method that is used in metabolomics studies. This unsupervised model uses (dis)similarity to group samples in data sets and encompasses successive pair-wise sample grouping based on predefined distance (Sumner *et al.*, 2003; Cook & Rutan, 2014; Alonso *et al.*, 2015; Young & Alfaro, 2016). The hierarchical clustering outcome is represented as a dendrogram (binary tree) which visually summarises the data (Sumner *et al.*, 2003; Putri *et al.*, 2013; Cook & Rutan, 2014; Madala *et al.*, 2014; Young & Alfaro, 2016). HCA is advantageous in that it allows researchers to visually interpret biological features in two-dimensional spaces especially, and for large sample number analysis (Putri *et al.*, 2013).

Although PCA, a descriptive model, is the basis of MVDA in metabolomics, which helps to visually explore trends and patterns in the data – giving an overview of the data, an additional method that allows classification and biomarker identification is required. OPLS-DA is a supervised linear regression and prediction method employed in the identification of class differences in a data matrix, using class information (Trygg *et al.*, 2007; Bylesjö, 2008; Bylesjö *et al.*, 2008; Fonville *et al.*, 2011; Tugizimana *et al.*, 2013; Madala *et al.*, 2014). OPLS-DA was developed as a modification of PLS-DA (Bylesjö *et al.*, 2006; Westerhuis *et al.*, 2010). The calculation between multivariate data and response variables with class information leads to generation of a regression model. Herein, predictive and orthogonal variation is explained

where, the first component(s) is predictive, describing the variation related to the treatment while the other components are orthogonal components describing variation not related to the treatment (Wiklund *et al.*, 2008; Westerhuis *et al.*, 2010). An OPLS-DA score plot is generated to visualise the discrimination between sample groups, and an OPLS-DA loadings plot for indicating shared features and extraction of discriminating variables that are statistically significant. OPLS-DA loadings plots such as the shared-and-unique-structures (SUS)-plot and S-plot are thus pivotal in the variable selection process (Wiklund *et al.*, 2008; Roux *et al.*, 2011; Worley & Powers, 2015; Ncube *et al.*, 2016). OPLS-DA differs from PCA in that it facilitates identification, extraction and interpretation of variables responsible for the discrimination between groups (Madsen *et al.*, 2010; Tugizimana *et al.*, 2013; Madala *et al.*, 2014). These statistically significant features are then annotated in order to link the results to the biological question (biological interpretation). Metabolite annotation is a critical step in any untargeted metabolic study (Johnson & Gonzalez, 2012; Misra *et al.*, 2017); and biological interpretation of data greatly depends on well-structured databases available for annotation (Tugizimana *et al.*, 2013).

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Chapter 3: Experimental Procedures

As mentioned in **Chapter 1**, to unravel the biochemistry underlying sorghum's responses to bacterial infection, the current study was designed to comprise two systems: (i) leave tissue and (ii) cell suspensions. For the former, young sorghum plants were treated by spraying a suspension of the bacterial pathogen *B. andropogonis* onto leaves; and for the latter sorghum cells suspensions were elicited with a lipopolysaccharide isolated and purified from *B. andropogonis*. The details of experimental design and procedures are provided in the following sections.

3.1. Plant leaf tissue system

3.1.1. Planting and growing of *Sorghum bicolor* plants

Sorghum seeds of the two South African commercial cultivars (cvs) NS 5511 (bitter or BT) and NS 5655 (sweet or ST) (Agricol, Pretoria, South Africa) were used for this study. More information regarding the two cultivars is listed in **Table 3.1**. The seeds were initially surface-sterilised by a sodium hypochlorite (jik): water solution (1: 2, v/v) before being placed in glass Petri dishes (with soaked paper towel) and incubated at 28 °C in the dark for 48 h to induce germination. All these above procedures were performed under strict sterile conditions. Following induced germination, the seedlings were planted in vermiculite for growth, under a 12 h fluorescence light (≈ 85 µmol m^{-2} s^{-2}) and 12 h dark cycle, mimicking outdoor day and light conditions. As indicated in the following paragraphs, the study was designed to monitor the responses for 9 days post-inoculation (d.p.i.); and at each time point (*i.e.* 1, 3, 5, 7 and 9 d.p.i.): 3 biological replicates with each replicate comprising 7 plants. Temperatures in the plant growth room were kept within the 22–27 °C range. During the plant growth period, watering was done regularly: at least 2 times a week using water-soluble chemical fertiliser (Multisol 'N', Culterra, Muldersdrift, RSA) dissolved in distilled water. All the plants were grown at the same time under the same environmental conditions (same quality and quantity of light, temperature and water volumes applied) as to minimise unwanted variation. **Figure 3.1** shows young sorghum plants at the three-leaf growth stage.

Table 3.1: Some characteristics of the *Sorghum bicolor* cultivars used for the plant tissue study (adapted from Department of Agriculture, Forestry and Fisheries, 2012 and Capstone seeds, Howick, South Africa, 2016).

Name	**NS 5511**	**NS 5655**
Other names	Bitter (BT)	Sweet (ST)
Type	Hybrid	Hybrid
Class	Malting class	Malting class
Grading	GH	GM
Condensed tannins	Present in high levels	Absent
Testa type	Dark testa with a bitter taste- undesirable to birds	Testa not dark
Seed colour	Red	Red
Plant height	~150 cm	~155 cm
Disease resistance rating to head smut, leaf disease and root rot (on 1-9 scale; 1= very resistant)	3	3

Figure 3.1: Healthy sorghum seedlings at the 3-leaf growth stage (~ 21 d after planting). The plant growth conditions were kept at ≈ 85 µmol m^{-2} s^{-2} light intensity for the 12 h light cycle, 12 h dark cycles and a 22–27 °C temperature range with regular plant watering (~2 times a week).

3.1.2. Bacterial culture and bacterial suspension preparation

Medium for bacterial culturing was prepared by dissolving 9.6 g nutrient broth (Merck, Johannesburg, RSA) in 600 mL (1:62.5 w/v) distilled water in a 1 L Erlenmeyer flask, followed by autoclaving and cooling at room temperature in a laminar flow hood, under strict sterile conditions. After cooling, the medium (pH 7.1) was inoculated using a 1.5 mL stock solution of the *B. andropogonis* strain 256 (BD 256) in glycerol (1 mL bacterial culture + 500 µL 80% glycerol), which was previously stored at − 80 °C. The initial bacterial stock solution was obtained from Plant Protection Research Institute (PPRI, Agriculture Research Council, Pretoria, RSA). Following inoculation, the bacterial culture was incubated overnight on a shaker at 28 °C and speed of 130 rpm. Bacterial cells were then harvested from the culture by means of centrifugation using a JA-10 fixed angle rotor (Beckman Coulter, Indianapolis, IN, USA) at 9 000 x *g* and at temperature of 4 °C for 20 min. The pellet was collected and the supernatant discarded. Phosphate buffered saline (PBS, Sigma, St. Louis, MO, USA), prepared by dissolving tablets of PBS in distilled water as per manufacturer's instructions (*i.e.* 1 tablet for every 200 mL). Initially the harvested bacterial cells (pellet) were resuspended in 2 mL PBS. The working (bacterial) suspension was subsequently prepared by serially diluting the above suspension (100 X to a volume of 800 mL), with PBS solution, until an optical density (OD) of 0.1 was obtained. To complete the preparation of the bacterial suspension, 800 µL (1:1000 v/v) of Insure pH buffer (Gouws and Scheepers (Pty) Ltd/ Plaaskem (Pty) Ltd, RSA) was added (as per manufacturer instructions) to improve wetting and spreading properties of the bacterial suspension.

3.1.3. Infection of the sorghum seedlings with bacterial suspension

At the 4-leaf growth stage (about 30 d after sowing), sorghum seedlings were treated by spraying the leaves equally and homogenously with the bacterial suspension (OD = 0.1), using a hand sprayer. After inoculum application, treated plants were incubated at 30 °C, in a high humidity environment, in darkness for 24 h. Following this incubation period, the plants were again exposed to the initial growth conditions mentioned in **section 3.1.1**. This study was designed to monitor the plant response to bacterial infection over time: 1, 3, 5, 7 and 9 d.p.i.. Thus, following the treatment, plant leaves from both cultivars were harvested at 1, 3, 5, 7 and 9 d.p.i. and at 1, 5 and 9 d.p.i. for the non-treated plant leaves (*i.e.* negative controls – not

sprayed). Leaves were cut from the plant and immediately stored at –80 °C to quench metabolic activity until the metabolite extraction steps could be performed.

3.2. Cell suspension system

Plant cell suspension cultures systems are used in various studies and their use has grown over the years. Applications of these systems include large scale biotechnological production of secondary metabolites (Dixon, 1999; Bourgaud *et al.*, 2001; Ramirez-Estrada *et al.*, 2016). The growing interest in plant suspension cultures is due to the advantages offered by such systems, which include shorter growth cycles than plant systems, reduced complexity, continuous availability of experimental material and rapid and increased experimental reproducibility due to the strictly controlled cell culture growing conditions (Ngara *et al.*, 2008; Allwood *et al.*, 2011). Furthermore, the biological material provided by cell suspension cultures is ideal for secondary metabolite biosynthetic pathways studies (Bourgaud *et al.*, 2001) and metabolomics studies of inducible defence responses (Tugizimana *et al.*, 2012).

3.2.1. Culturing and harvesting bacterial cells for LPS isolation and purification

An overnight (small scale) bacterial culture prepared as described in **section 3.1.2** was used for inoculation of large scale cultures. Three 5 L Erlenmeyer flasks, containing 3 L of nutrient broth, were each inoculated with 200 mL of the overnight (small scale) bacterial culture, under strict sterile conditions. To ensure optimum bacterial growth, the large scale cultures were incubated at 30 °C on a rotating shaker at 100 rpm for 14 d. Growth of the large scale bacterial cultures was repeated several times (cycles) to ensure larger quantities of starting material (bacterial cells) for LPS isolation and purification. Harvesting of bacterial cells was performed by centrifugation at 13 000 ×*g*, 4 °C for 20 min, using a JA-10 rotor (fixed-angle centrifuge; Beckman Coulter, Indianapolis, IN, USA) with 6 x 400 mL centrifuge tubes capacity. The pellets were collected each time and the supernatants discarded. Harvesting of bacterial cells was followed by freeze-drying for 48 h before the LPS isolation and purification steps (**section 3.2.2**).

3.2.2. LPS isolation and purification

LPS was extracted from the bacterial cells biomass cultured as indicated in section **3.2.1**, using the hot phenol extraction protocol as described by De Castro *et al.*, (2010). The particular extraction used for this study involves lysing bacterial cells and partitioning of the LPS into the aqueous phase for a maximum LPS yield. Harvested *B. andropogonis* (BD 256) bacterial cells (**section 3.2.1**) were initially washed with 248 mL of 0.9% sodium chloride solution and then lyophilised. Following freeze-drying, 33 g of dried bacterial cells were suspended in 578 mL (1: 17.5 w/v) warm water (65-70 ºC) and an equal volume of preheated (65-70 ºC) 90% phenol was added to the mixture. The above procedure was performed on a heated stirrer. The mixture was kept at 65 ºC for 15 min and then placed on ice until the temperature dropped to 10 ºC. This was followed by centrifuging the obtained emulsion at 10 000 ×*g* for 30 min at 4 ºC. The upper water phase was separated from the milky interphase and bottom phenol phase, and kept aside. To the remaining phases (phenol and interphase) an equal volume of warm water was added and the extraction procedure was repeated thrice – collecting the water phases each time. Following extraction, the combined water phases were concentrated to 200 mL using a rotary evaporator set at 55 ºC, and followed by dialysis (7000 molecular weight cut-off membrane, 'Snakeskin' dialysis tubing, Pierce, Thermo Scientific, Rockford, IL, USA) for 3-5 d – changing the water frequently to remove traces of phenol. The dialysed water phase was centrifuged again at 10 000 ×*g* for 20 min followed by freeze-drying.

For LPS purification, enzymatic digestion of the RNA that co-extracted into the water phase, was used. Dried extracts were dissolved in 30 mL (1: 40 w/v) distilled water and treated with 0.1 mg RNase (Sigma-Aldrich, Steinheim, Germany) and incubated at 37 ºC for 2 h. Proteinase K (0.1 mg) (Sigma-Aldrich, Steinheim, Germany) was then added and the mixture was incubated at 37 ºC for 2 h. Following incubation, 30 mL of phenol was added in order to denature the enzymes and the mixture was centrifuged at 10 000 ×*g* for 15 min to obtain the water phase. This was dialysed for 3 d with frequent changes of distilled water and then lyophilised. The mass of purified LPS obtained thereof was determined and sent for characterisation to the Department of Chemical Sciences, University of Napoli Federico II, Naples, Italy (Di Lorenzo and Molinaro, unpublished).

3.2.3. LPS-specific SDS-PAGE analysis

Isolated and purified LPS from both *B. andropogonis* and *B. cepacia* (as the marker) were prepared as follows for SDS-PAGE analysis: LPS stocks of 1 mg/mL were diluted with a 2X sample buffer in a ratio of 1:1. The sample buffer was made up of 0.05 M Tris (hydroxymethyl) aminomethane, at pH 6.8 (Merck, Darmstadt, Germany), 2% Sodium Dodecyl Sulpahte (SDS) (Sigma-Aldrich, St Louis, USA), 10% sucrose (Merck, Darmstadt, Germany) and 0.05% bromophenol blue (Saarchem, Muldersdrift, RSA). The samples were then heated for 5 min at 100°C. A 12.5% SDS-PAGE gel was prepared by casting a 15% resolving gel [2.6 mL distilled water, 4.67 mL of 30% monomer solution (acrylamide/bisacrylamide in 29:1 ratio, Merck, Darmstadt, Germany), 2.5 mL 1.5 M Tris at pH 8.8, 100 µL 10% SDS, 100 µL 10% ammonium persulphate (APS, Merck, Darmstadt, Germany) and 10 µL TEMED (Merck, Hohenbrunn, Germany)] into an assembled gel cassette system, followed by additionally pouring water-saturated butanol on top of the resolving gel, and leaving the gel to polymerise. Following polymerisation, the water-saturated butanol was discarded and rinsed off with distilled water. A 4% stacking gel [4 mL distilled water, 3.3 mL 30% monomer solution (acrylamide/ bisacrylamide in 29:1 ratio), 2.5 mL 1.5M Tris at pH 6.8, 100 µL 10% SDS, 120 µL 10% ammonium persulphate (APS), 50 µL TEMED] was then poured on top of the resolving gel and a teflon comb was inserted, and the gel left to polymerise. The comb was removed following polymerisation of the stacking gel and the gels cassette was placed into the electrophoresis tank and 1X tank buffer added to it. The 1 X tank buffer was prepared from a 10 X buffer [10 g SDS + 30.3 g Tris + 1.44.1 g glycine (Sigma-Aldrich, St Louis, USA) in 1 L dH_20] by diluting 100 mL of the 10 X with 900 mL distilled water. Fifteen µL of the LPS samples prepared as described above were then loaded onto the gel and electrophoresed using the Hoefer Scientific miniVE vertical electrophoresis system (Hoefer, Richmond CA, USA) at voltage of 300 volts, constant current of 12 mA for separating gel and 25 mA for stacking gel. Upon completion of electrophoresis, a silver staining procedure was performed according to the Fomsgaard *et al.* (1990) and Tsai & Frasch, (1982) protocols for visualisation of the various LPS moieties/bands.

For silver staining, 0.7% periodic acid (H_5IO_6, Merck, Darmstadt, Germany) in 40% ethanol and 5% acetic acid (Rochelle Chemicals, Johannesburg, RSA) was added for the oxidation of the LPS moieties/bands in the gel and left for 20 min (no prior fixation method was required). Thereafter the gel was washed for 5 min in distilled water (this was repeated thrice). A staining

solution [of concentrated ammonium hydroxide (Rochelle Chemicals, Johannesburg, RSA), 0.1 M sodium hydroxide, 20% (w/v) silver nitrate ($AgNO_3$ (Merck, Darmstadt, Germany), freshly prepared] was added to the gel which was continuously agitated for 10 min. This was followed by washing of the gel with distilled water for 5 min (this was done thrice). A developer solution [200 mL distilled water, 10 mg citric acid (Sigma-Aldrich, St Louis, USA), 37% formaldehyde (Sigma-Aldrich, St Louis, USA)] was poured onto the gel and left until the bands developed. To terminate the developing process, 10% acetic acid stop solution was added and left for 15 min, followed by the washing the gels in distilled water for 30 min (this was done twice).

3.2.4. Sorghum cell culture establishment and growth

In the cell suspension study, Sweet White sorghum variety (Agricol, Pretoria, RSA) was used for callus development. Seeds were prepared and germinated as previously described by Ngara *et al.*, (2008). Initiation of callus development was then carried out by plating germinated sorghum shoots on full strength solid Murashige and Skoog (MS) media [0.8% (w/v) phytoagar and 3% (w/v) sucrose, pH 5.8] including MS vitamins and added phytohormones [3 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.5 mg/L 1-naphthaleneacetic acid (NAA)], at 25 ºC in the dark with continuous monitoring of growth over 4 weeks (Ngara *et al.*, 2008; Ngara & Ndimba, 2011). After successful callus development, the callus was sub-cultured in MS media (same composition as above) and left to develop for 3 weeks before equally distributing the callus clumps into small Erlenmeyer flasks containing half-strength liquid MS medium (same composition as above but without agar). The cell cultures were incubated on a horizontal shaker with continuous agitation at a speed of 130 rpm at room temperature, with 12 h light and 12 h dark cycles. Once successfully established in the liquid medium (14 d), the cells were sub-cultured into a number of flasks containing freshly prepared MS media (plus MS hormones and vitamins), and incubated on the horizontal shaker with the same growth conditions (described above) (**Figure 3.2**). The above procedures were performed under strict sterile conditions and the cell suspensions sub-cultured every 14 d.

Figure 3.2: Sorghum cell suspension cultures obtained after the initial cell suspension sub-culturing. After successful establishment of cell suspension from callus, the cell suspensions were sub-cultured into MS medium with MS hormones and vitamins followed by an incubation for 7 d with continuous shaking at 130 rpm, to obtain the cell suspension cultures represented in the figure.

3.2.5. Elicitation of sorghum cell suspension with *Burkholderia andropogonis* LPS

B. andropogonis LPS (isolated and purified as described in **section 3.2.2**) was prepared for sorghum cell suspension elicitation by initially dissolving LPS in MS medium at room temperature to give a 10 mg/mL final stock solution. Sorghum cell suspensions *i.e.* 100 mL from each of the five 250 mL Erlenmeyer flasks were first combined and mixed to ensure a homogeneous mixture of cells. Equal aliquots (25 mL) were then redistributed into pre-weighed, sterile 50 mL Falcon tubes – with three biological replicates for each condition. Treatment of the cell suspensions was performed by adding 250 µL of the prepared LPS stock solution, so as to results in a final concentration of 100 µg/mL in each of the tubes. For the negative control conditions, no LPS was added. The treated and non-treated cell suspensions were then placed horizontally on an orbital shaker and incubated at 130 rpm and 25 °C. A time study (0, 12, 18, 24 and 30 hours post-inoculation, h.p.i.) was conducted to monitor the response of the cells to treatment over time. Treated cells were harvested at 12, 18, 24 and 30 h.p.i., while control cells were at 0 h.p.i., using centrifugation in a bench top swinging bucket centrifuge at 5100 rpm and 4 ºC for 25 min. Pellets and supernatants were separated and

immediately stored at –80 °C until both intracellular and extracellular metabolite extraction steps could be performed.

3.3. Metabolite extraction and pre-analytical sample preparation

For plant leaf tissue, metabolites were extracted from treated and non-treated (NS 5655/sweet/ST, and NS 5511/ bitter/BT) plants using 80% cold methanol in a ratio of 1:15 (w/v). For sorghum cell suspensions, intracellular metabolites were extracted using 100% cold methanol in a ratio of 1:2 (w/v), working at 4 °C. In both cases, following addition of extraction solvent, the mixture was homogenised using an Ultra Turrax homogenizer and sonication using a probe sonicator (Bandelin Sonopuls, Germany) set at 55% power for 15 s. The homogenates were centrifuged at 5000 ×*g* for 25 min at 4 °C, (swinging-bucket centrifuge; Beckman Coulter, Brea, CA USA) and supernatants were kept. To concentrate the extracts, the supernatants (of each sample) were evaporated under vacuum to 1 mL using a rotary evaporator set at 55 °C, and then evaporated to complete dryness with a speed vacuum concentrator (Eppendorf, Merck, Johannesburg, RSA) set at 45 °C. Extracellular metabolites were extracted as follows; the supernatant (media) obtained after centrifugation was first lyophilized and the obtained material was kept for re-suspension. The dried extracts (from plant leaf tissue and intra-, and extracellular fractions of cultured cells) were then re-suspended in 50% UHPLC-grade methanol (Romil Pure Chemistry, Cambridge, UK) in a 1:10 m/w ratio. This was followed by filtering samples through 0.22 µm nylon syringe filters into UHPLC glass vials fitted with 500 µL inserts. The filtered extracts were capped and kept at –20 °C until analysed.

3.4. Ultrahigh-performance liquid chromatography-high definition mass spectrometry (UHPLC-HDMS) analyses

UHPLC and high-definition mass spectrometry analyses were performed on a Waters Acquity UHPLC coupled in tandem to a Waters photodiode array (PDA) detector and SYNAPT G1 Q-TOF mass spectrometer (Waters Corporation, Milford, MA, USA). Chromatographic separation of the methanolic extracts was done using a Waters HSS T3 C18 column (150 mm × 2.1 mm ×1.8 µm), in a column oven maintained at 60 °C. Gradient elution was carried out with a binary solvent system consisting of 0.1% formic acid (Sigma-Aldrich, St. Louis, MO, USA) in MilliQ water (solvent A) and 0.1% formic acid in acetonitrile (Romil Pure Chemistry,

Cambridge, UK) (solvent B) at a flow rate of 0.4 mL/min. The initial conditions were 2% B and were maintained for 1 min. The gradient was ramped to 95% B at 15 min and maintained for 2 min, and then changed to the initial conditions at 18 min, followed by a 2 min equilibration time of the column. The total chromatographic run time was 20 min and the injection volume was 2 µL for plant tissue extracts and 4 µL for cell suspension extracts. Each sample was analysed in triplicate to account for any analytical variability.

High definition mass spectrometry (MS) was performed on a Waters SYNAPT G1 Q-TOF system operated in V-optics, and with an electrospray ionisation (ESI) source interface. Leucine encephalin (50 pg/mL) was used as a reference calibrant to obtain typical mass accuracies between 1 and 3 mDa. The MS data were acquired in both positive and negative modes, with a capillary voltage of 2.5 kV, sampling cone at 30 V, extraction cone at 4 V, cone gas flow 50 L h^{-1} and desolvation gas flow 550 L h^{-1}. The source temperature was 120 °C and the desolvation temperature 450 °C. A scan time of 0.1 s was used with a 100-1000 Da mass range. The data were acquired with different collision energies (MS^E) 10–50 eV to obtain as much structural information as possible for detected compounds. The MassLynx software (V4.1 SCN 872, Waters Corporation Milford, MA, USA) was used to control the hyphenated system and perform initial data manipulation.

3.5. Data processing and multivariate data analyses

Raw data, both ESI negative and positive, obtained from UHPLC-HDMS, were extracted using MassLynx^{TM} XS software and processed with MarkerLynx software (Waters Corporation, Manchester, UK). Data pre-processing included peak picking, peak alignment, noise filtering, peak area integration and normalisation. Varying software parameters were used for data processing. The data matrices (samples = N, and Rt-*m/z* variables with integrated peak areas) obtained from MarkerLynx processing were exported into SIMCA 14, Omics skin (Umetrics, Umea, Sweden) for statistical analyses. The data were Pareto-scaled before principal component analysis (PCA), hierarchical cluster analysis (HCA), and orthogonal partial least squares discriminant analysis (OPLS-DA). The generated models were validated using different methods (as described in the respective sections in **Chapter 4**; **section 4.3** and **Chapter 5**; **section 5.2**).

3.5.1. XCMS online data processing and analysis

The XCMS (various forms of chromatography–mass spectrometry) online package was additionally used for further analyses of LC-MS data. This bioinformatics open-source tool based on the R language and accessible on the web address: https://xcmsonline.scripps.edu, was employed complementary to MarkerLynx and SIMCA-based analyses. This was essential for the aim of comprehensive coverage of the metabolites associated with treatment of sorghum plants with *B. andropogonis* (Wei *et al.*, 2012; Benton *et al.*, 2015; Tugizimana *et al.*, 2015; Mahieu *et al.*, 2016; Ncube *et al.*, 2016). The following parameters were used in the XCMS analysis: feature detection was set with *m/z* deviation of 15 ppm; minimum peak width of 5 and maximum peak width of 20; Rt correction was attained using Orbiwarp method; alignment set at 0.5, Rt deviation at 5 s, *m/z* window at 0.015; for statistical test the unpaired parametric t-test was performed, with *p*-value threshold of 0.05 and a fold change threshold of 1.0; annotation with *m/z* absolute error of 0.002.

3.6. Metabolite annotation

In untargeted metabolomic studies, metabolite annotation still remains a challenging task. As such, many metabolites remain uncharacterised owing to the complexity in biological systems. (Camacho *et al.*, 2005; Johnson & Gonzalez, 2012; Courant *et al.*, 2014; Misra *et al.*, 2017). Although, MS can attain accurate mass and produce compounds fragments, to provide more information about the compound structure, manual interpretation of large numbers of fragmentation spectra is time-consuming and labour intensive making it nearly impractical. In addition, different molecules may produce similar spectra (Scheubert *et al*., 2013 Hufsky *et al*., 2014). However, several approaches have been put into place over the years (including novel approaches) to aid in identification of unknown metabolites, as clearly reviewed by Misra *et al.* (2017). Noteworthy, the availability of information and defined pathways in databases still remains a limiting factor thereof. On the other hand, authentic reference standards also facilitate definitive and confidence in metabolite identification through comparing MS data and retention time of compounds of interest in the study with these standards (acquired under the same experimental settings) (Sumner *et al.*, 2007; Gowda & Djukovic, 2014; Misra & van der Hooft, 2016). While ideal, the limitations with regards to authentic reference standards include excessive costs (when multivarious standards are needed) (Clifford & Madala, 2017) and commercial unavailability for some plant metabolites (Plazonić *et al.*, 2009; Hossain *et al.*,

2010). With consideration to the above mentioned factors, the annotated metabolites are reported with a degree of certainty (**Table 3.2**) set by the Chemical Analysis Working Group (CAWG) – part of the Metabolomics Standard Initiative (MSI) (Sumner *et al.*, 2007).

Table 3.2: The metabolite annotation/identification reporting levels laid by the Chemical Analysis Working Group (CAWG) (adapted from Finnegan, 2012).

Level	Metabolite annotation	Level of evidence
1	Confidently identified compounds	Comparison of two or more orthogonal properties with an authentic reference standard analysed under similar experimental settings.
2	Putatively annotated compounds	Here, no reference standard is used. Physicochemical properties and/or spectral similarities with public/commercial spectral libraries is the basis for annotation.
3	Putatively compound classes characterised	Characteristic physicochemical properties of a chemical class of compounds, or spectral similarity to known compounds of a chemical class is the basis of annotation.
4	Unknown compounds	Based on spectral data, metabolites can still be differentiated and quantified, even though unidentified or unclassified.

In this study the first step in metabolite annotation involved running data matrices obtained after MarkerLynx processing (raw data) on the Taverna workbench (www.taverna.org.uk) for PUTMEDID_LCMS metabolite identification workflows. These comprise correlation analysis, metabolic feature annotation and metabolite annotation (Brown *et al.*, 2009). The resulting metabolite identities generated (together with adducts and molecular formulae) were then confirmed with the aid of fragmentation patterns. Here, accurate masses obtained from the Waters SYNAPT G1 Q-TOF system were used to generate empirical formulae. If the mass difference between measured and calculated mass was at or below 5 mDa, the corresponding formulae was selected and queried against available online databases such as Dictionary of Natural Products (DNP) (dnp.chemnetbase.com), ChemSpider (www.chemspider.com), PubChem (www.pubchem.ncbi.nlm.nih.gov), PlantCyc (www.plantcyc.org), SorgCyc (www.sorgcyc.org) and KNApSAcK (http://kanaya.naist.jp/knapsack_jsp/top.htm). Parameters such as isotopic fit (iFit) and double bond equivalent (DBE) were also taken into consideration in the selection of the formulae. Literature available on sorghum metabolites was also used to aid in metabolite annotation/confirmation. Thus, annotation of metabolites in this study was done at level 2 of the Metabolomics Standards Initiative (MSI), see **Table 2** (Sumner *et al.*, 2007).

List of references

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Chapter 4: Results and Discussion, part I: Metabolomic analyses of the defence response of two *Sorghum bicolor* cultivars to infection by *Burkholderia andropogonis*

4.1. Bacterial leaf stripe symptom progression and evaluation in treated *Sorghum bicolor* plants

Plant diseases resulting from bacterial or fungal infection usually manifest symptoms distinctive to the pathogen on the infected part of the plant (*e.g.* leaves). Thus, upon diagnosis of plant diseases, the evaluation of symptoms is a critical step as an indicator of disease severity and/or host resistance (Riley *et al.*, 2002; Vidaver & Lambrecht, 2004; Bock & Nutter, 2011; Matsunaga *et al.*, 2017). Following treatment of NS 5511 (BT = bitter) and NS 5655 (ST = sweet) sorghum cvs with *B. andropogonis*, the progression of bacterial leaf stripe symptoms on the leaves was monitored over time.

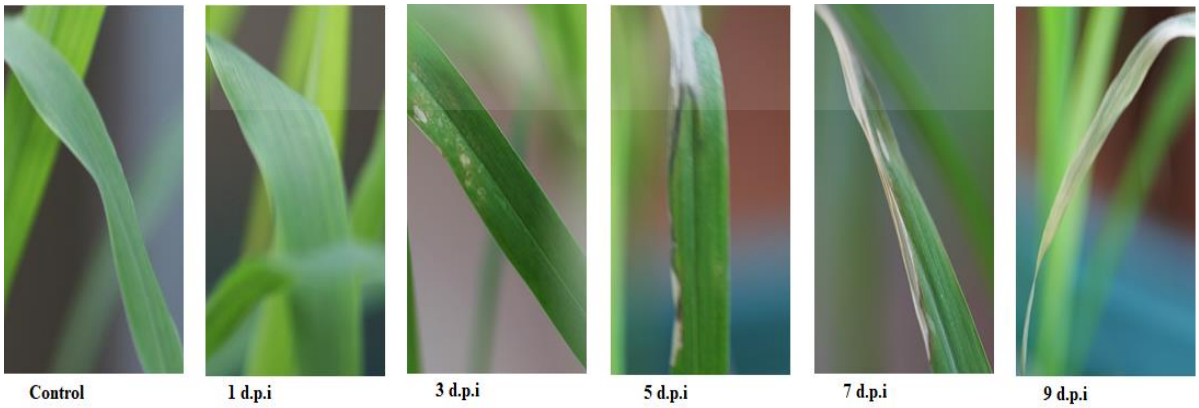

Figure 4.1: Symptom progression on sorghum leaves (NS 5511 cv) subsequent to infection with *B. andropogonis*. The symptoms are compared to the non-treated plants (control). On 1 d.p.i. (days post inoculation) no symptom development can be noticed. However, at 3 d.p.i. small tan lesions start to show and progress (lesion elongation) over the days to a point where most parts of the leaves are covered in lesions and drying of leaves can be noticed (9 d.p.i.).

Figure 4.2: Symptom progression on sorghum leaves (NS 5655 cv) subsequent to infection with *B. andropogonis.* The symptoms are compared to the non-treated plants (control). On 1 d.p.i. (days post inoculation) no symptom development can be noticed. However, at 3 d.p.i. small tan lesions start to show and progress (lesion elongation) over the days to a point where most parts of the leaves are covered in lesions and drying of leaves can be noticed (9 d.p.i.). Note, a similar symptom progression trend can be noticed as for NS 5511 cv (**Figure 4.1**).

The visual inspection of symptom development on sorghum leaves (on both NS 5511and NS 5655 cvs), following the bacterial infection, showed typical bacterial leaf stripe symptoms (**Figures 4.1** and **4.2**) as previously reported in sorghum plants infected with *B. andropogonis* (Claflin *et al*., 1992; Ramundo & Claflin, 2005). Small linear tan lesions appeared on the inoculated plant leaves – which progressively elongated with time, nearly encompassing the entire leaf surface (**Figures 4.1** and **4.2**), and indicative of the disease progression. Initially, no visible symptoms were observed at 1 d.p.i. for both cvs. The plants, however, started to display symptoms at 3 d.p.i. (**Figures 4.1** and **4.2**). Appearance of lesions is one of the first visible indicators of host plant–pathogen interactions (Kachroo & Kachroo, 2009). As described by Bagsic *et al.* (1995), the leaf stripes (lesions) develop as a result of pathogen invasion of parenchymatous tissue. Additionally, the subsequent launching of defence responses following perception of a pathogen leads to events such as, the onset of programmed cell death and production of defence-related secondary metabolite production – in order to limit the pathogen or infection from spreading to other parts of the leaf/plant (Zvereva & Pooggin, 2012; Balmer *et al.*, 2013; Poloni & Schirawski, 2014; Andolfo & Ercolano, 2015; Tugizimana, 2017). As stated by Mizuno *et al.*, (2016) the development of pigmented lesions is regarded as an action set to enhance plant resistance.

Furthermore, a disease severity rating was done based on the percentage of leaf area covered in symptoms **(Table 4.1)**. Generally, both sorghum cvs (NS 5511 and NS 5655) showed similar symptom development, as infographically depicted in **Figures 4.1** and **4.2**. However, NS 5655

cv displayed signs of wilting at an earlier stage relative to the former. The NS 5511 cv, thus, appeared to be more resistant to the bacterial infection based on the symptomatology. This is in accordance with Tugizimana (2017) who reported the NS 5511 cv as more resistant. The symptom progression displayed, therefore, showed a cultivar- and time-dependant response to *B. andropogonis*.

Table 4.1: Bacterial leaf stripe disease severity rating in NS 5511 (BT) and NS 5655 (ST) sorghum cvs.

Disease severity index according to cultivar		
Days post inoculation (d.p.i.)	**NS 5511 (BT)**	**NS 5655 (ST)**
1	1	1
2	1	1
3	2	2
4	2-3	3
5	3	3
6	3-4	4
7	4	4-5
8	4	5
9	5-6	5-6

*Severity ratings index was defined as follows:
1 = no lesions;
2 = 1 - 10% lesions.
3 = 11 -25% lesions.
4 = 26 -50% lesions and signs of wilting.
5 = 51 - 75% lesions and ≤50% wilting.
6 = 76-100% lesions and >50% wilting.

4.2. Ultra high performance liquid chromatography-high definition mass spectrometry (UHPLC-HDMS) analyses of extracts derived from non-treated and treated sorghum plants

Aqueous-methanol extracts from both treated and non-treated plant leaf tissue of the two *S. bicolor* cvs, NS 5511 and NS 5655, were analysed on an UHPLC-HDMS platform as described in **Chapter 3** (**section 3.4**). These extracts displayed an inherent multidimensionality emerging from the complex physicochemical characteristics of the sample constituents, thus making chromatographic separation necessary (Giddings, 1995; Schure, 2011; Tugizimana *et al*., 2013; Tugizimana *et al.*, 2016). When optimised, LC allows the separation of a high number of metabolites, thereby reducing the analytical complexity of the analytes before MS analysis; thus, enhancing MS and subsequent data analyses (Allwood *et al.*, 2008; Allwood & Goodacre, 2010; Tugizimana *et al.*, 2013).

From LC-MS analyses of the aqueous-methanol extracts, the resulting chromatography infographically shows the complexity of the extracts, *i.e.* comprising a mixture of polar, mid-

polar and non-polar compounds (with more of the compounds being mid-polar) and differences across the samples. For the latter, differential observations include variation in peak intensities and presence/absence of peaks across samples; suggesting differential metabolic profiles: *i.e.* time-related **(Figure 4.3** and **S 4.1-4.3)** and cultivar-related metabolic responses **(Figure 4.4** and **S 4.4),** because of bacterial infection. Some notable chromatographic differences in peak intensities and peak population can be visually observed in the 9-9.5 min (highlighted in red) and 12.5-15.0 min (highlighted in green) regions of **Figure 4.3**, respectively – reflecting induced treatment-related and time-related differential metabolic profiles in sorghum plants following the bacterial infection.

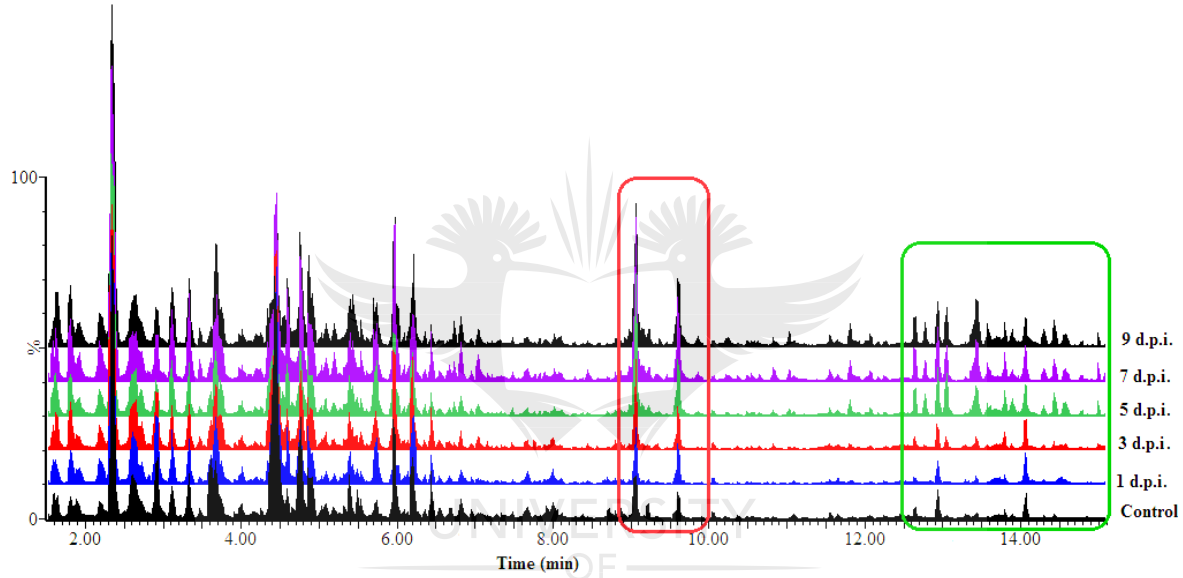

Figure 4.3: UHPLC-MS BPI chromatograms for ESI negative data of extracts derived from sorghum NS 5511 (BT) cv responding to *B. andropogonis* infection. Sample extracts were prepared from plant material harvested at the indicated time intervals. Variation in the displayed chromatograms, linked to treatment- and time-related metabolic changes can be visually observed from control (non-treated) sample *vs* treated samples (1 - 9 d.p.i.) chromatograms.

Furthermore, differential responses of the cultivars (cv-related response) to bacterial treatment could be observed across samples (**Figure 4.4**). A closer visual inspection of the chromatograms generated from NS 5511 *vs* NS 5655 extracts depicts variation in peak population and peak intensities amongst the two cv samples: this is revealed at 3 d.p.i. and 5 d.p.i. time points in the highlighted region *i.e.* 12.6-14.5 min Rt range.

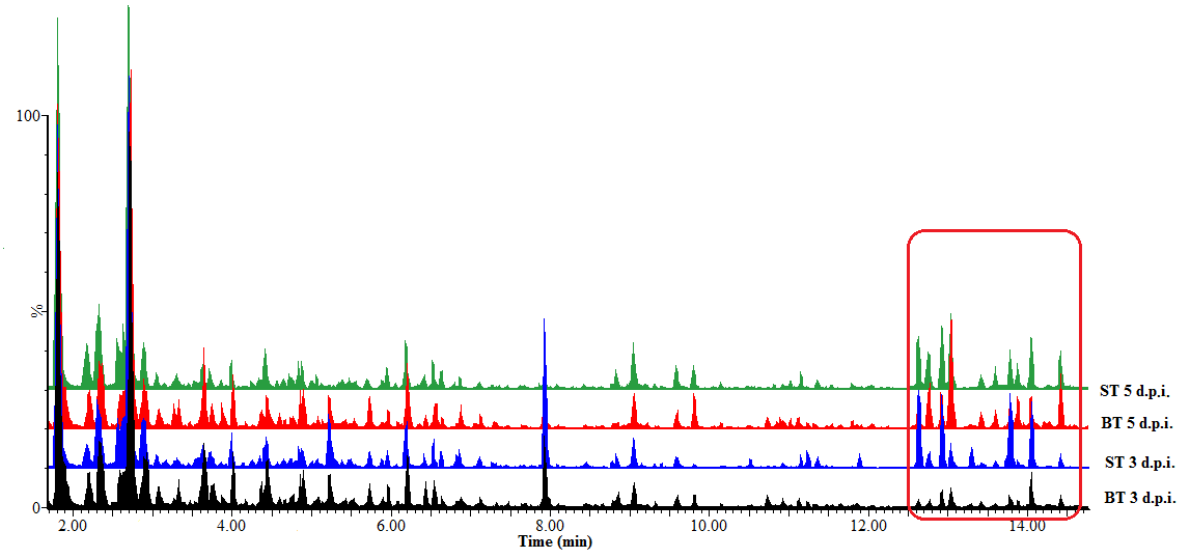

Figure 4.4: Comparative UHPLC-MS BPI chromatograms for ESI positive data of extracts derived from sorghum NS 5511 (BT) vs NS 5655 (ST) cvs responding to *B. andropogonis*. NS 5511 treated sample chromatograms at 3 and 5 d.p.i. shown against NS 5655 treated samples at the same time period, visually showing cultivar-related metabolic changes.

4.3. Multivariate data analyses: statistical description and explanation of variation in the acquired LC-MS data

To further explore and explain the differences visually observed on the chromatograms, statistical analyses were performed. As mentioned in **Chapter 2** (**section 2.4**), non-targeted metabolomics, as a high-throughput 'omics' approach, enables the measurement of thousands of metabolites simultaneously. The results thereof are information-rich and highly complex data. This, therefore, requires various chemometrics/multivariate data analysis tools which are a better fit in handling confounding and covariance patterns (between and within variables) – which is not normally feasible with traditional statistical methods (Trygg *et al.,* 2007; Tugizimana *et al.,* 2012, 2013, 2014; Saccenti *et al.,* 2013). To highlight the metabolic changes induced by *B. andropogonis* treatment, multivariate data analyses methods were employed to mine the collected multidimensional data: reducing the dimensionality of the data, exploring the data so as to unravel structures, patterns within the data; and identifying variables explaining sample groupings in the multivariate space. These methods thus allowed the extraction of relevant biological information related to the study at hand (Goodacre *et al.*, 2004; Reshetova *et al.,* 2014; Tugizimana *et al.*, 2013, 2015).

As mentioned in **Chapter 3**, unsupervised methods namely PCA and HCA were applied, following data pre-processing and pre-treatment. PCA provided a non-biased reduction of data dimensionality and facilitated the identification of trends and patterns in the data, thus giving an overview thereof (Madsen *et al.*, 2010; Ncube *et al.*, 2016; Misra & van der Hooft, 2016), in relation to bacterial treatment. The computed PCA models revealed time- and cv-related sample clustering. All the calculated PCA models were mathematically robust and had a reliable predictive accuracy – explaining more than 50% of the total variation in matrix X, with a predictive ability greater than 50% according to seven-fold cross-validation. The PCA scores plots (**Figures 4.5 A** and **S 4.5-4.7 A**) show treated samples clustering separately from the control samples. A time trend clustering can also be seen when the scores plot is coloured based on time points (**Figures 4.5 B** and **S 4.5-4.7 B**). PCA models computed based on cvs **(Figures 4.6 A-B** and **S 4.8 A-B)** showed a clear separation between the NS 5511and NS 5655 sorghum cvs. The observed sample clustering described above reflects the differences in metabolite profiles across the samples and between the two sorghum cvs respectively and thus, induced differential metabolic responses to *B. andropogonis* treatment.

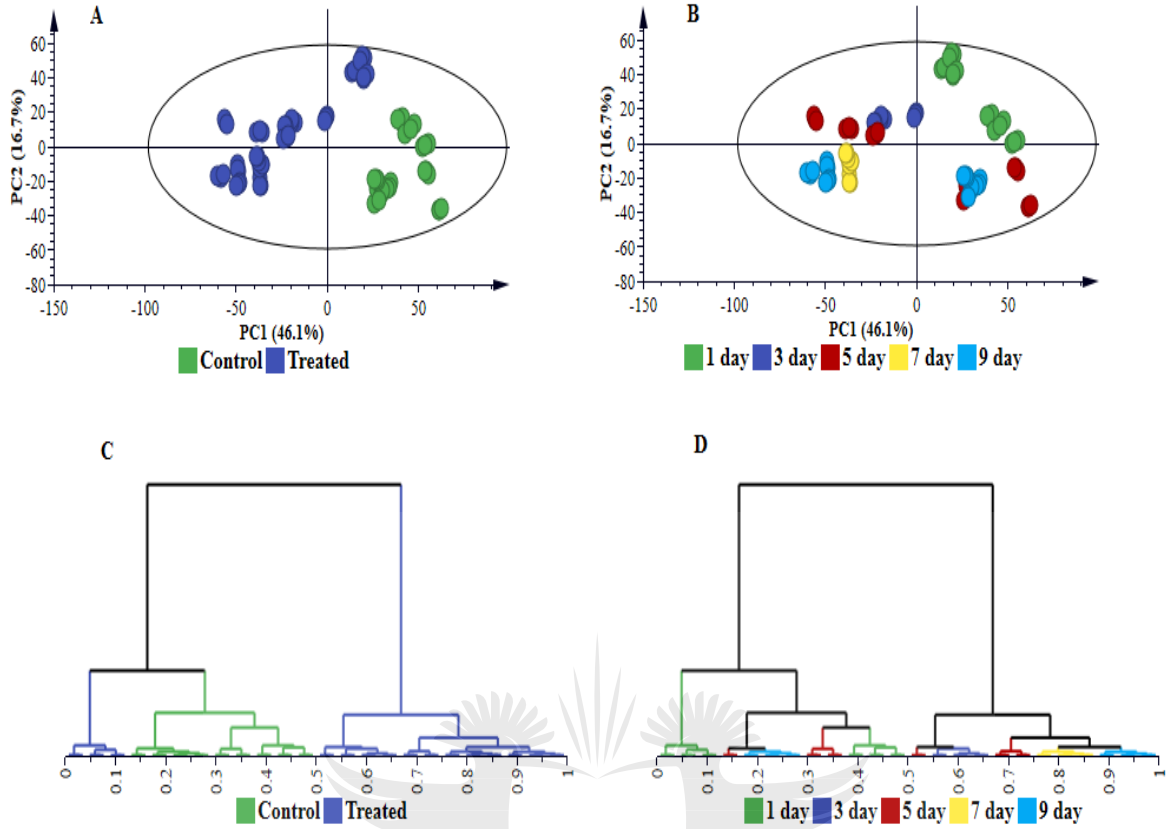

Figure 4.5: PC and HC analyses of the ESI negative data for sorghum NS 5511 (BT) extracts. (A & B): A 11-component model, explaining 84.2% variations in Pareto-scaled data, X, and the amount of predicted variation by the model, according to cross-validation, is 74.1%. **A** and **B** is the same scores plot: with **A** coloured according to treatment and **B** is coloured according to time. This two-dimensional scores space, spanned by the first two PCs, reveals treatment-related sample clustering (treated = blue, controls/non-treated = green) and also time-related clustering, respectively. **Note**: to determine the group (control/treated) to which the time-related clusters in **B** and **D** belong, link to the corresponding positions in **A** and **C**, respectively (this applies to all the PCA and HCA plots). **(C & D):** HCA dendrograms computed from low dimensional data (**PC scores plot**). The models which correspond to **A** and **B** respectively were computed using 11 vectors/components, explaining 84.2% of the total variation in X matrix. **C** displays treatment-related relationships amongst the samples; treated (blue) samples are clearly separated from the non-treated samples (green) except for a few samples, while **D** displays time-related relationships.

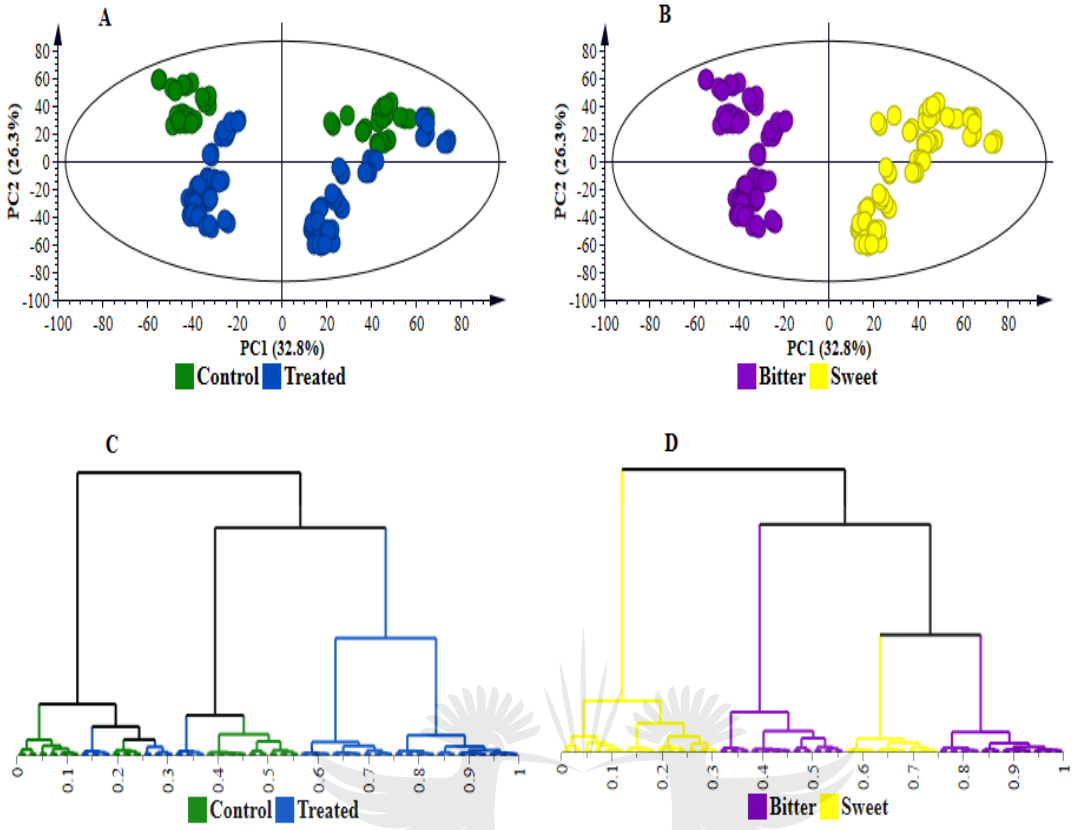

Figure 4.6: PC and HC analyses of the ESI negative data for sorghum NS 5511 (BT) and NS 5655 (ST) extracts. **(A & B):** 15-component models, explaining 86.0% variations in Pareto-scaled data, X, and the amount of predicted variation by the model, according to cross-validation, is 79.6%. **A** and **B** is the same scores plot, with **A** coloured according to condition and **B** coloured according to cv. This two-dimensional scores space, spanned by the first two PCs, reveals treatment-related sample clustering (treated = blue, controls/non-treated = green) **(A)** and also cv-related clustering **(B)**. **(C & D):** HCA dendrograms computed from low dimensional data (**PC scores plot**). The models were computed using 15 vectors/components, explaining 86.0% of the total variation in X matrix. **C** displays treatment-related relationships amongst the samples; treated (blue) samples from both cvs are clearly separated from the non-treated samples (green) except for a few samples, while **D** displays cv-related relationships.

HCA was applied on low-dimensional data generated from the PC analyses – as a complimentary data exploration method, that also offers a visual summary of the data (Putri *et al.*, 2013; Madala *et al.*, 2014). Moreover, the analyses facilitated in revealing hidden structures in the data (*e.g.* any samples subgroupings) as well as further scrutiny/confirmation of the trends displayed by the PC analyses. Here, dendrograms were computed from the metabolite space using the Ward linkage method to reveal sample classifications (Hall, 2011; Madala *et al.*, 2014; Tugizimana, 2017). Descriptively, similar sample clustering to PC analyses was shown by the HCA models (**Figure 4.5 C-D** and **S 4.5-4.7 C-D**). Here, two major clusters of treated samples clustering separately from the non-treated samples were displayed (except for treated day 1 which clustered with the non-treated sample) by both cvs in ESI negative data.

Additionally, the dendrograms displayed a trend associated with different time points. Dendrograms corresponding to the PCA scores plot, generated based on cvs, reveal treatment **(Figure 4.6 C** and **S 4.8 C)** and cv-related **(Figure 4.6 D** and **S 4.8 D)** sub-groupings respectively. Bacterial infection, thus, induced differential metabolic reprogramming in *S. bicolor* plants; with cv-related nuances.

To complement the descriptive view provided by PCA and HCA modelling, a supervised statistical tool namely OPLS-DA together with XCMS online analysis were applied to evaluate and explain the metabolic changes (uncovered by unsupervised tools) of sorghum plants responding to the bacterial infection. The OPLS-DA method aided in identifying class differences in the data matrix, facilitating the identification, extraction and interpretation of the features responsible for the class differences (Bylesjö *et al.*, 2006; Madsen *et al.*, 2010; Putri *et al.*, 2013; Gowda & Djukovic, 2014; Hill & Roessner, 2015; Tugizimana *et al.*, 2016).

Generated OPLS-DA score plots **(Figure 4.7 A** and **S 4.9 A, 4.11 A, 4.13 A)** show evident classification of samples *i.e.* the samples are clearly grouped into two distinct classes of treated (blue) and non-treated (green) samples. These calculated OPLS-DA models were statistically significant models (with CV-ANOVA *p*-value less than 0.05), and computed to separate multivariate relationships into: predictive variation (related to bacterial infection) and orthogonal variation (unrelated to bacterial infection) (Tugizimana *et al.*, 2014, 2015, 2016). Furthermore, the OPLS-DA models were validated using various diagnostic tools, to determine how well these binary classification models performed and to also rule out model-overfitting in the supervised modelling (Madsen *et al.*, 2010; Hrydziuszko & Viant, 2012; Bartel *et al.*, 2013; Naz *et al.*, 2014; Alonso *et al.*, 2015). These validation steps are regarded as necessary when handling highly dimensional datasets from LC–MS (Yin & Xu, 2014).

Distance to the model in space X (DModX) was used as a tool for detecting moderate outliers. DModX plots **(Figure 4.7 B** and **S 4.9 B, 4.11 B, 4.13 B)** show a few moderate outliers *i.e.* observations whose DModX value is above the Dcrit (critical value of DModX; 0.05). To check the performance of OPLS-DA as a binary classifier, the receiver operator characteristic (ROC) plot was employed. ROC plots **(Figure 4.7 C** and **S 4.9 C, 4.11 C, 4.13 C)** graphically summarised a perfect discrimination depicted by the OPLS-DA models (binary classifier) *i.e.* computed models were perfect classifiers – as revealed by the high sensitivity and specificity (~ 100%) of the ROC curve (Tugizimana *et al.*, 2016; Tugizimana, 2017). Moreover,

permutation testing (**Figure 4.7 D** and **S 4.9 D, 4.11 D, 4.13 D**), as a powerful tool in measuring the performance of a model, was carried out to determine whether the performance of the computed binary classification (OPLS-DA) models were statistically significant. The permutation tests revealed that the computed OPLS-DA models were statistically significant and that in the separation of classes, none of the permutated models showed a better performance than that of the original models (Hendriks *et al.*, 2011; Alonso *et al.*, 2015; Young & Alfaro, 2016; Tugizimana, 2017).

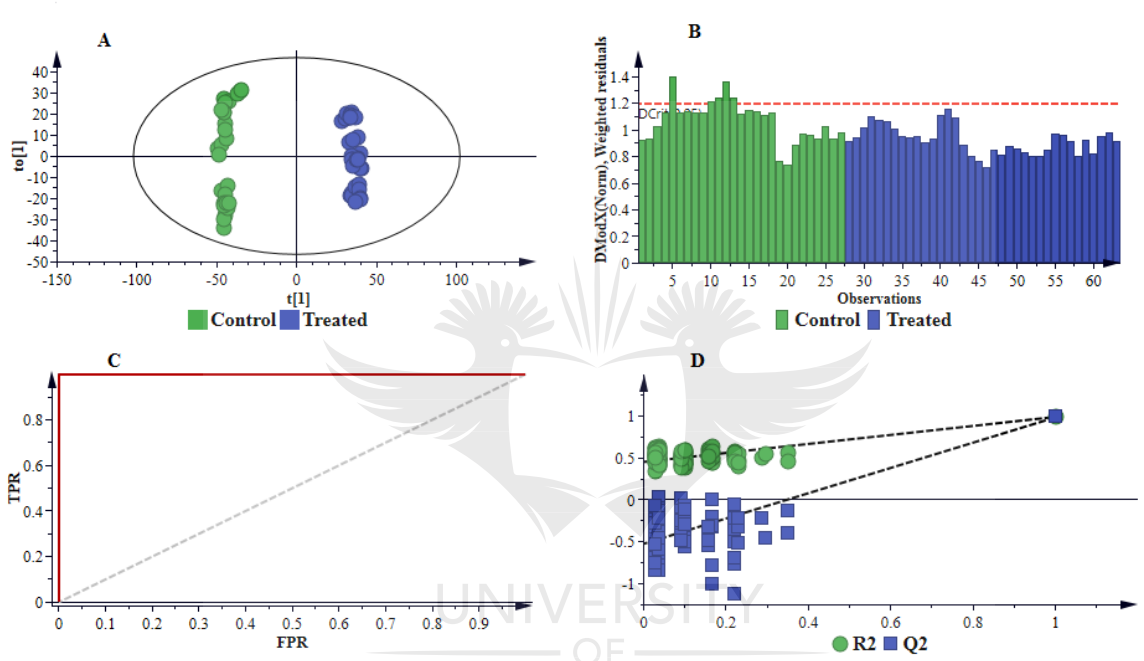

Figure 4.7: Supervised multivariate analyses of the ESI negative UHPLC-MS data for NS 5511 (BT) cv extracts (excluding QCs). (A): The OPLS-DA score plot shows grouping of control *vs* treated for all samples. This model comprises 1 predictive component and 2 orthogonal components (R^2X= 67.2%, R^2Y= 99.3% and Q^2= 98.7%). **(B):** A distance to the model in space X (DModX) plot showing moderate outliers (above the dashed red line, Dcrit) in the OPLS-DA scores plot. (**C**): A representative receiver operator characteristic (ROC) plot summarising the performance of OPLS-DA (a binary classification method). **(D):** The response permutation test plot (n=100) of the OPLS-DA model in **A**, clearly separating control from treated plant samples.

To facilitate the extraction of features responsible for the discrimination between treated and non-treated samples, variable selection methods such as the OPLS-DA loading S-plot and XCMS cloud plot were used. Features significantly contributing to the model, with $|p[1]| \geq 0.05$ and $|p(corr)| \geq 0.5$ were extracted from the OPLS-DA loading S-plot (*e.g.* the variable highlighted in red) (**Figure 4.8 A** and **S 4.10 A, 4.12 A, 4.14 A**) for downstream metabolite identification.

The XCMS online was additionally used to avoid statistical bias in variable selection – an alternative method to identify significant variables explaining the patterns described by PCA. Applying different methods (other than MarkerLynx-SIMCA) aids in the identification of additional biologically important variables (Patti *et al.*, 2012, 2013; Gowda *et al.*, 2014). The interactive Cloud plots (**Figure 4.8 B** and **S 4.10 B, 4.12 B, 4.14 B**), generated from XCMS online analyses, show discriminant features positively correlated to bacterial infection on the upper part of the plot (in green) and those negatively correlated to bacterial infection on the bottom part of the plot (in red). The *m/z* of each feature is presented by the *y*-coordinate and Rt by the *x*-coordinate. The size of the displayed bubbles relates to the Log fold change of the feature and the feature colour intensity indicates statistical significance of the fold change – calculated by a Welch *t* test with unequal variances, where features with low *p* values are less intense than features with high *p* values. Moreover, a black outline on the bubbles indicates that the features have hits on the METLIN databases (Patti *et al.*, 2013; Gowda *et al.*, 2014; Tugizimana *et al.*, 2015).

The significance of the variables extracted from the S-plot was statistically validated using variable importance in projection (VIP) plots and dot plots. VIP scores evaluation obviated variable selection bias and helped describe the importance of the variables to the model. On the VIP plot, variables with a score more than 1 are considered significant (Putri *et al.*, 2013; Gowda & Djukovic, 2014; Finnegan *et al.*, 2016; Tugizimana *et al.*, 2016; Liang *et al.*, 2017) and an increase in VIP score correlates to increased significance (Tugizimana *et al.*, 2015; Finnegan *et al.*, 2016). Therefore, from the VIP plots the variables with a score greater than 1 were chosen for metabolite annotation. **Figure 4.8 C** (and **S 4.10 C, 4.12 C, 4.14 C**) show some of the selected variables (*e.g.* highlighted in red) validated using VIP plots prior to metabolite annotation. For further evaluation of the selected variables, dot plots (**Figure 4.8 D** and **S 4.10 D, 4.12 D, 4.14 D**) were generated to investigate how discriminant the variables are. For instance, no overlap between the treated and control sample can be seen from the selected *m/z* = 327.21480 variable in **Figure 4.8 D**, also highlighted in the S-plot, and VIP plots, thus suggesting that the particular feature contributed strongly to the discrimination (Tugizimana *et al.*, 2016).

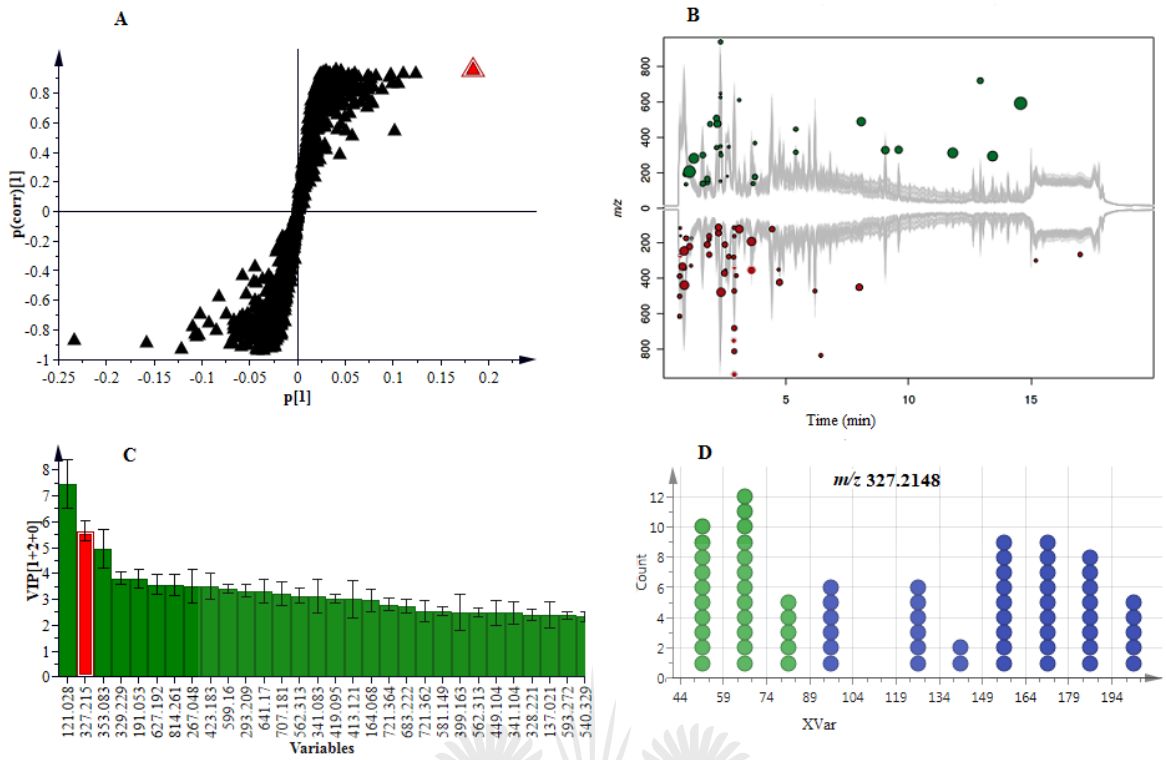

Figure 4.8: OPLS-DA modelling analyses of the UHPLC-MS ESI negative data for NS 5511 (BT) cv extracts (excluding QCs). (A): The OPLS-DA loading S-plot displays the discriminating features (ions) that explain the clustering (sample grouping) observed in the OPLS-DA scores plot with the features in the top right quadrant positively correlated to the treatment and those in the bottom left quadrant negatively correlated to the treatment. The loading S-plot comprises 1 predictive component explaining 47.1% of the total variation and 2 orthogonal components explaining 20.1% of the total variation. **(B):** XCMS cloud plot displaying discriminating ions; green for the treated samples and red for non-treated samples. **(C)**: A VIP plot summarising the importance of some of the variables in the projection of the model. A VIP value >1 is significant/important in the projection and higher score values indicate an increase in significance of the variables. **(D):** Dot plot of the selected variable *m/z* **327.21480** from S-plot (in red; also marked on the VIP plot) showing no overlap between control and treated groups, the variable thus strongly discriminates the two groups.

Following validation of the extracted signatory variables considered as important contributors to the class discrimination, metabolite annotation was carried out. As described in **Chapter 3** (**section 3.6**), these variables relating to metabolic changes following bacterial treatment were annotated at MI-level 2 of the Metabolomics Standards Initiative (MSI) and are listed in **Table 4.2**. The fold changes and *p*-values of the various metabolites presented in the **Table 4.2** were obtained from the models constructed from all control samples against all the treated samples of the NS 5511 and NS 5655 cvs.

Table 4.2: Annotation of discriminatory metabolites belonging to various chemical classes, related to *Burkholderia andropogonis*-induced metabolic reprogramming in *Sorghum bicolor* plants. Following UHPLC-MS, the metabolites were selected and extracted from OPLS-DA S-plots and annotated at MI-level 2 (in both positive and negative ionisation modes). The reported fold changes for cvs NS 5511 and NS 5655 were obtained from an OPLS-DA model of control (1-9 d.p.i.) *vs* all treated samples. Common synonyms of the metabolites are bracketed.

Metabolites	*m/z*	Rt (min)	Adduct	Ion mode	Molecular formula	NS 5511		NS 5655		Metabolite class
						p-value	Fold change	*p*-value	Fold change	
L-Phenylalanine	180.092	2.59	$[M-H\_NH_3]^-$	neg	$C_9H_{11}NO_2$	0.159	0.9	0.002	0.9	Amino acid
L-Tyrosine	182.081	1.13	$[M+H]^+$	pos	$C_9H_{11}NO_3$	1.77E-31	0.5	1.94E-31	0.4	Amino acid
L-Tryptophan	205.097	2.69	$[M+H]^+$	pos	$C_{11}H_{12}N_2O_2$	1.87E-27	0.6	2.67E-15	0.5	Amino acid
N,N-dihydroxy--tyrosine	234.038	8.04	$[M-H\_Na]^-$	neg	$C_9H_{11}NO_5$	8.94E-18	0.1	1.25E-14	0.1	Amino acid
5-Methyl-tryptophan	277.072	5.71	$[M+H\_NaNa]^+$	pos	$C_{12}H_{14}N_2O_2$	8.83E-33	0.3	0.804	0.9	Amino acid
Benzoic acid	121.028	4.46	$[M-H]^-$	neg	$C_7H_6O_2$	1.76E-20	0.6	0.603	0.7	Benzoates
Gallic acid monohydrate	187.095	6.74	$[M-H]^-$	neg	$C_7H_8O_6$	1.75E-23	4.7	1.53E-15	3.8	Benzoates
Dhurrin	334.090	2.6	$[M+H\_Na]^+$	pos	$C_{14}H_{17}NO_7$	2.49E-29	0.4	2.51E-27	0.4	Cyanogenic glycoside
Apigenin	269.007	7.05	$[M-H]^-$	neg	$C_{15}H_{10}O_5$	0.838	1.0	0.000	1.5	Flavonoid
Protocatechuic acid 4-O-beta-glucoside	315.069	5.41	$[M-H]^-$	neg	$C_{13}H_{16}O_9$	5.24E-07	1.5	1.67E-11	2.1	Flavonoid
Tricin	329.066	8.92	$[M-H]^-$	neg	$C_{17}H_{14}O_7$	6.62E-07	0.7	0.091	0.8	Flavonoid
Beta-D-apiofuranosyl-(1->6)-D-glucose	330.141	2.59	$[M+NH_3]^+$	pos	$C_{11}H_{20}O_{10}$	4.28E-34	0.4	2.04E-29	0.4	Flavonoid
Sophoraflavanone B	341.137	2.50	$[M+H]^+$	pos	$C_{20}H_{20}O_5$	8.06E-34	0.4	4.38E-27	0.4	Flavonoid
4',5,5'-Trihydroxy-2',3,6,7,8-pentamethoxyflavone	419.095	5.69	$[M-H]^-$	neg	$C_{20}H_{20}O_{10}$	1.64E-24	3.1	1.31E-16	3.8	Flavonoid
Sophoraflavanone G	423.182	5.46	$[M-H]^-$	neg	$C_{25}H_{28}O_6$	4.59E-11	0.6	1.29E-05	0.7	Flavonoid
Apigenin-8-*C*-glucoside (vitexin)	431.099	5.55	$[M-H]^-$	neg	$C_{21}H_{20}O_{10}$	7.57E-05	1.4	0.347103	1.1	Flavonoid
Apigetrin (apigenin 7-O-glucoside)	431.098	6.33	$[M-H]^-$	neg	$C_{21}H_{20}O_{10}$	0.238	1.0	0.001	0.8	Flavonoid
Naringenin 7-O-beta-D-glucoside (prunin)	433.114	5.91	$[M-H]^-$	neg	$C_{21}H_{22}O_{10}$	1.44E-16	2.3	5.76E-18	4.6	Flavonoid
Luteolin 7-O-glucoside	447.091	5.71	$[M-H]^-$	neg	$C_{21}H_{20}O_{11}$	4.44E-13	1.6	0.799	1.0	Flavonoid
Quercetin 3-O-rhamnoside (quercitrin)	447.092	4.61	$[M-H]^-$	neg	$C_{21}H_{20}O_{11}$	9.02E-17	1.8	8.37E-17	3.8	Flavonoid
Pentahydroxychalcone 4'-O-glucoside	449.108	4.57	$[M-H]^-$	neg	$C_{21}H_{22}O_{11}$	7.50E-11	1.4	5.49E-19	2.1	Flavonoid
Apigenin 8-C-xyloside-6-C-glucoside (vicenin-3)	563.139	5.09	$[M-H]^-$	neg	$C_{26}H_{28}O_{14}$	0.672	1.1	3.88E-08	1.2	Flavonoid
Apigenin 6-C-xyloside-8-C-glucoside (vicenin-1)	563.140	4.87	$[M-H]^-$	neg	$C_{26}H_{28}O_{14}$	6.91E-11	1.2	0.008	1.1	Flavonoid
Vitexin 2"-O-rhamnoside	577.154	5.32	$[M-H]^-$	neg	$C_{27}H_{30}O_{14}$	1.43E-15	1.5	7.43E-14	1.5	Flavonoid

Table 4.2: continued..

Apigenin 7-O-neohesperidoside (rhoifolin)	577.156	6.06	$[M-H]^-$	neg	$C_{27}H_{30}O_{14}$	1.52E-07	1.1	0.488	1.0	Flavonoid
Unknown flavonoid	581.149	4.33	$[M-H]^-$	neg	$C_{26}H_{30}O_{15}$	7.67E-25	2.5	1.75E-15	2.8	Flavonoid
Luteolin 7-O-neohesperidoside	593.150	5.51	$[M-H]^-$	neg	$C_{27}H_{30}O_{15}$	2.20E-15	1.3	0.037	0.9	Flavonoid
Apigenin-6,8-di-C-glucoside (vicenin-2)	593.151	4.45	$[M-H]^-$	neg	$C_{27}H_{30}O_{15}$	0.637	1.0	8.60E-06	1.3	Flavonoid
Quercetin-3-rhamnoside-7-rhamnoside	595.165	4.51	$[M-H]^-$	neg	$C_{27}H_{32}O_{15}$	2.87E-09	1.4	4.17E-10	1.5	Flavonoid
Quercetin rutinoside (rutin)	609.146	5.43	$[M-H]^-$	neg	$C_{27}H_{30}O_{16}$	1.22E-19	2.1	3.98E-10	2.1	Flavonoid
Hesperidin	609.181	4.80	$[M-H]^-$	neg	$C_{28}H_{34}O_{15}$	0.236	1.1	0.000	0.9	Flavonoid
Unknown flavonoid	611.158	3.10	$[M-H]^-$	neg	$C_{27}H_{32}O_{16}$	4.59E-09	1.2	3.07E-08	1.4	Flavonoid
Naringenin 7-O-neohesperidoside (naringin)	625.180	3.33	$[M-H\_HCOOH]^-$	neg	$C_{27}H_{32}O_{14}$	0.000	1.1	3.10E-06	0.7	Flavonoid
7-O-Methylvitexin 2"-O-beta-L-rhamnoside	637.177	6.21	$[M-H\_HCOOH]^-$	neg	$C_{28}H_{31}O_{14}$	0.466	1.0	0.563	1.0	Flavonoid
4-Hydroxycoumarin	161.024	1.87	$[M-H]^-$	neg	$C_9H_6O_3$	3.91E-12	0.6	0.017	0.8	HCA
p-Coumaric acid	163.039	3.65	$[M-H]^-$	neg	C_9H8O_3	0.064	0.9	0.953	0.9	HCA
Caffeic acid	179.034	4.35	$[M-H]^-$	neg	$C_9H_8O_4$	1.68E-07	0.5	0.083	0.6	HCA
Ferulic acid	193.048	4.01	$[M-H]^-$	neg	$C_{10}H_{10}O_4$	1.95E-12	0.8	1.25E-07	0.8	HCA
Sinapoyl alcohol	209.074	6.72	$[M-H]^-$	neg	$C_{11}H_{14}O_4$	1.48E-15	2.4	1.07E-16	3.1	HCA
Coniferyl acetate	221.081	7.42	$[M-H]^-$	neg	$C_{12}H_{14}O_4$	nd	nd	3.47E-10	8.2	HCA
Coumaryl acetate	237.078	2.66	$[M-H]^-$	neg	$C_{11}H_{12}O_3$	2.06E-09	3.7	0.002	0.7	HCA
2-O-Caffeoylglyceric acid	267.048	4.38	$[M-H]^-$	neg	$C_{12}H_{12}O_7$	1.48E-10	0.5	0.017	0.7	HCA
4-Coumaroylquic acid	337.051	3.29	$[M-H]^-$	neg	$C_{16}H_{18}O_8$	1.54E-30	3.8	6.41E-26	3.6	HCA
Caffeic acid hexose	341.083	6.17	$[M-H]^-$	neg	$C_{15}H_{18}O_9$	3.44E-07	0.7	0.003	0.8	HCA
4-Caffeoylquinic acid	353.091	3.58	$[M-H]^-$	neg	$C_{16}H_{18}O_9$	4.75E-23	0.3	2.60E-05	0.5	HCA
1-O-Feruloyl-beta-D-glucose	355.102	4.06	$[M-H]^-$	neg	$C_{16}H_{20}O_9$	3.21E-24	0.2	4.17E-18	0.1	HCA
4-Coumaroylagmatine	359.113	2.63	$[M-H\_HCOONa]^-$	neg	$C_{14}H_{20}N_4O_2$	0.0464	0.9	1.09E-07	0.8	HCA
3-Feruloylquinic acid	367.099	3.75	$[M-H]^-$	neg	$C_{17}H_{20}O_9$	5.98E-09	1.3	0.002	1.2	HCA
Sinapoyl aldehyde	371.130	6.53	$[M+H]^+$	pos	$C_{17}H_{22}O_9$	4.70E-29	0.3	3.58E-22	0.4	HCA
2-O-Caffeoylglucarate	371.062	2.05	$[M-H]^-$	neg	$C_{15}H_{16}O_{11}$	0.167	0.3	nd	nd	HCA
1-O-Coumaroyl-beta-D-glucose	371.097	4.26	$[M-H\_NaNa]^-$	neg	$C_{15}H_{18}O_8$	0.001	1.1	0.442	1.0	HCA
Sinapoyl-(S)-malate	385.078	3.74	$[M-H\_HCOOH]^-$	neg	$C_{15}H_{16}O_9$	1.56E-10	0.8	0.066	0.6	HCA
1-O-Sinapoyl-beta-D-glucose	385.113	5.16	$[M-H]^-$	neg	$C_{17}H_{22}O_{10}$	7.38E-09	0.8	0.000	0.9	HCA
Feruloylserotonin	395.100	3.65	$[M-H\_NaNa]^-$	neg	$C_{20}H_{20}N_2O_4$	0.793	1.0	1.43E-10	0.3	HCA
1,3-O-Coumaroyl-feruloylglycerol	413.121	9.03	$[M-H]^-$	neg	$C_{22}H_{22}O_8$	9.84E-12	0.4	4.47E-17	0.5	HCA
Sinapaldehyde glucoside	415.123	4.44	$[M-H\_HCOOH]^-$	neg	$C_{17}H_{22}O_9$	7.13E-16	0.7	9.48E-07	0.8	HCA

Table 4.2: Continued..

1,3-O-Diferuloylglycerol	443.132	9.22	$[M-H]^-$	neg	$C_{23}H_{24}O_9$	4.20E-11	0.4	2.53E-26	0.3	HCA
Caffeic acid derivative	475.143	1.92	$[M-H]^-$	neg	$C_{20}M_{28}O_{13}$	1.81E-15	1.5	2.56E-17	2.5	HCA
1,2-bis-O-Sinapoyl-beta-D-glucoside	591.166	6.19	$[M-H]^-$	neg	$C_{28}H_{32}O_{14}$	0.547	1.0	0.105	1.0	HCA
Indole-3-acrylic acid/ N-AC-indole-3-carboxyaldehyde	188.076	2.71	$[M+H]^+$	pos	$C_{11}H_9NO_2$	2.12E-27	0.5	1.85E-18	0.5	Indole
Methyl indole-3-acetate	190.085	2.69	$[M+H]^+$	pos	$C_{11}H_{11}NO_2$	4.47E-11	0.5	4.33E-09	0.5	Indole
Indole-3-pyruvate	202.051	7.89	$[M-H]^-$	neg	$C_{11}H_9NO_3$	3.07E-18	1.9	5.79E-13	2.5	Indole
Indolylmethylthiohydroximate	273.032	3.20	[M-H_HCOONa]	neg	$C_{10}H_{10}N_2OS$	0.275	1.2	0.033	1.5	Indole
6-Hydroxy-indole-3-acetyl-valine	289.119	3.95	$[M-H]^-$	neg	$C_{15}H_{18}N_2O_4$	1.83E-09	1.7	0.469	0.9	Indole
Indole-3-acetyl-leucine	333.120	3.25	$[M+H_NaNa]^+$	pos	$C_{16}H_{20}N_2O_3$	1.45E-07	4.1	0.001	1.9	Indole
DIMBOA-Glc	372.093	1.58	$[M-H]^-$	neg	$C_{15}H_{19}NO_{10}$	0.001	0.8	9.77E-14	0.4	Indole
Indole-3-yl-acetyl-myo-inositol L-arabinoside	468.152	3.13	$[M-H]^-$	neg	$C_{21}H_{27}NO_{11}$	0.000	1.3	0.117	0.9	Indole
Isocitric acid	191.018	1.10	$[M-H]^-$	neg	$C_6H_8O_7$	5.97E-14	2.3	1.64E-09	1.6	Carboxylic acid
Octadecatetraenoic acid	275.200	13.44	$[M-H]^-$	neg	$C_{18}H_{28}O_2$	1.64E-17	4.1	1.20E-11	3.6	Fatty acid
16-Hydroxypalmitate	293.209	13.43	$[M-H_Na]^-$	neg	$C_{16}H_{31}O_3$	8.38E-19	4.9	9.55E-13	4.1	Fatty acid
15-Hydroxylinoleic acid	295.226	14.3	$[M-H]^-$	neg	$C_{18}H_{32}O_3$	1.38E-18	3.6	8.76E-14	3.4	Fatty acid
10,16-Dihydroxypalmitate	309.204	10.24	$[M-H_Na]^-$	neg	$C_{18}H_{30}O_4$	9.76E-14	8.0	6.75E-11	8.1	Fatty acid
Dihydroxy-octadecadienoic acid	311.220	11.81	$[M-H]^-$	neg	$C_{18}H_{32}O_4$	5.87E-17	4.9	2.18E-11	6.0	Fatty acid
11,12,13-Trihydroxy-9,15-octadecadienoic acid	327.215	9.06	$[M-H]^-$	neg	$C_{18}H_{32}O_5$	1.95E-32	2.7	1.30E-05	1.5	Fatty acid
9,12,13-Trihydroxy-10-octadecenoic acid	329.229	9.60	$[M-H]^-$	neg	$C_{18}H_{34}O_5$	3.23E-27	2.5	3.95E-17	2.0	Fatty acid
Salicylic acid	137.031	3.69	$[M-H]^-$	neg	$C_7H_6O_3$	4.29E-12	1.3	0.091	3.9	Phytohormone
Salicylic acid 2-O-beta-D-glucoside	299.074	1.62	$[M-H]^-$	neg	$C_{13}H_{16}O_8$	7.21E-17	1.7	9.28E-24	1.9	Phytohormone
Jasmonoyl-L-isoleucine	406.200	4.16	$[M-H_HCOOK]^-$	neg	$C_{18}H_{29}NO_4$	2.94E-12	0.5	5.03E-20	0.1	Phytohormone
Dihydrozeatin-9-N-glucoside-O-glucoside	634.197	2.80	$[M-H_NaHCOONa]^-$	neg	$C_{22}H_{35}N_5O_{11}$	0.001	0.8	4.22E-12	2.8	Phytohormone
Zeatin riboside	436.124	3.16	$[M+H_HCOOK]^+$	pos	$C_{15}H_{21}N_5O_5$	1.35E-28	0.3	3.27E-22	0.4	Phytohormone
Chorismic acid	225.040	2.61	$[M-H]^-$	neg	$C_{10}H_{10}O_6$	nd	nd	0.076	0.9	Shikimate
Caffeoylshikimic acid	335.076	4.64	$[M-H]^-$	neg	$C_{16}H_{16}O_8$	2.89E-17	0.3	3.84E-11	0.3	Shikimate/ HCA
Unidentified	432.273	7.95	–	pos	$C_{20}H_{37}N_3O_7$	2.66E-35	0.3	0.019	20.4	Unknown
Unidentified	489.269	8.08	–	neg	$C_{24}H_{42}O_{10}$	4.66E-26	3.4	6.03E-15	6.3	Unknown
Unidentified	518.317	13.03	–	pos	$C_{21}H_{47}N_3O_{11}$	3.11E-15	4.6	3.66E-14	3.4	Unknown
Unidentified	543.206	6.82	–	neg	$C_{25}H_{36}O_{13}$	0.000	1.3	0.035	1.1	Unknown

nd; not detected in the cv; *p*-value refers to significance level of a metabolite.; Fold change was calculated by dividing the average of the metabolite intensity in replicate samples of treated by the average of the metabolite intensity in replicate samples of control, a value ≥1 represents an increase (metabolite is higher in the treated samples than in the control) and value <1 represents a decrease. (metabolite is higher in the control and treatment led to decrease in levels). HCA; hydroxycinnamic acid

Metabolite annotation was achieved with the aid from the Taverna workbench (www.taverna.org.uk), databases such as Dictionary of Natural Products (DNP) (dnp.chemnetbase.com), ChemSpider (www.chemspider.com), PubChem (www.pubchem.ncbi.nlm.nih.gov), PlantCyc (www.plantcyc.org), SorgCyc (www.sorgcyc.org) and KNApSAcK (http://kanaya.naist.jp/knapsack_jsp/top.htm) and available literature.

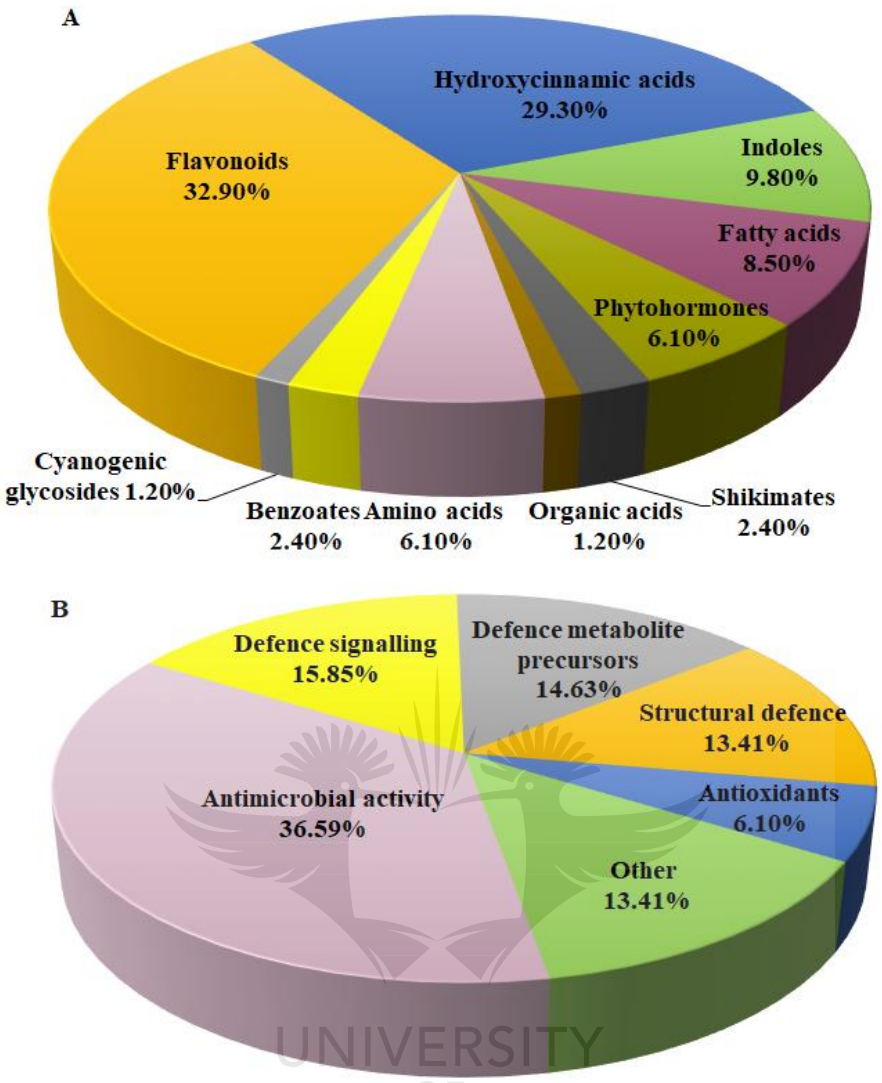

Figure 4.9: Classification of the putatively identified signatory metabolites in extracts of sorghum plants responding to infection by *B. andropogonis*, according to the chemical classes (A) and primary/prominent functions in defence (B). A total of 82 plant metabolites were putatively identified in sorghum extracts. **(A):** Illustrates the chemical diversity of metabolites potentially contributing to defence against *B. andropogonis* (flavonoids and hydroxycinnamic acids - two major classes). **(B):** Due to some metabolites possessing more than one function, grouping was based on the known primary/prominent role in plant defence.

The visual inspection of symptoms and chromatographic analyses results, further investigated using various statistical tools, evidently suggested the metabolic reprogramming in sorghum plants induced by *B. andropogonis* treatment. As previously mentioned in **Chapter 2** (**section 2.4.1**), LC-MS-based untargeted metabolomics facilitated annotation and analysis of an array of chemically diverse metabolites, representing a wide range of metabolic pathways, associated with sorghum response to bacterial infection **(Table 4.2** and **Figure 4.9 A**). Chemical classification (**Figure 4.9 A**) highlighted two major classes *i.e.* flavonoids and hydroxycinnamic acids, stipulating the significance of these metabolites in sorghum defences.

In addition, the annotated metabolites demonstrated to possess various defence-related functions as summarised in **Figure 4.9 B**. For example, challenged sorghum plants activated both structural and chemical defences, to counteract pathogen infection (as similarly summarised in **Chapter 2**; **Figure 2.6**). The diversity of the metabolites, arising from different metabolic pathways (particularly amino acid, fatty acid, shikimic acid, phenylpropanoid and flavonoid metabolic pathways) showed an intricate and dynamic network of the sorghum defence arsenal towards *B. andropogonis* in resistance and disease suppression (Bollina *et al.*, 2010; Wang *et al.*, 2012; Balmer *et al.*, 2013; Bigeard *et al.*, 2015; Rochat, 2016). Moreover, it reflected the significant genetic diversity and extensive adaptive abilities of sorghum (Salzman *et al.*, 2005). The metabolic fluctuations because of bacterial treatment as well as the functional roles of the annotated metabolites in defence responses are detailed in the following section. Relative quantities, expressed as fold changes, of the putatively identified metabolites were used to provide measurable evaluation of metabolic changes and to give a comprehensive picture of metabolic reprogramming in *S. bicolor* plants triggered by *B. andropogonis* treatment.

4.4. Metabolic reprogramming in the primary and secondary metabolism of *Sorghum bicolor* following *Burkholderia andropogonis* infection.

4.4.1 The role of aromatic amino acids in pathogen-induced stress responses

The treatment of sorghum with *B. andropogonis* resulted in changes in amino acid metabolism (primary metabolism). Of interest are changes in the three aromatic amino acids, L-tyrosine, L-tryptophan and L-phenylalanine. Relative quantitative analyses revealed a decrease of these amino acids in the respective levels (fold change < 1; **Table 4.2**) in treated plants across time (time-related changes; 1-9 d.p.i.) as well as cultivar-related differences in metabolite levels (data not shown). The role of metabolic pathways of distinct amino acids in the regulation of defence responses in pathogen-challenged plants has been demonstrated in several studies (López-Gresa *et al.*, 2010; Zeier, 2013; Gunnaiah & Kushalappa, 2014; Pasquet *et al.*, 2014; Andolfo & Ercolano, 2015; Cuperlovic-Culf *et al.*, 2016). Aromatic amino acids synthesised *via* the shikimic acid pathway (chorismic acid acting as their initiator metabolite) are central to plant metabolism – serving as precursors in the synthesis of a range of secondary metabolites

and phytohormones, with plant defence functions (Tzin & Galili, 2010; Du Fall & Solomon, 2011; Tohge *et al.*, 2013; Ng *et al.*, 2016).

Phenylalanine is an important precursor for phenylpropanoid, flavonoid (Ayabe *et al.*, 2010; Bollina *et al.*, 2010; Beelders *et al.*, 2014; Edwards, 2016) and cyanogenic glycoside secondary metabolites, whereas tyrosine and tryptophan are involved in cyanogenic glycosides and indole metabolite synthesis respectively (Tzin & Galili, 2010; Du Fall & Solomon, 2011; Piasecka *et al.*, 2015). Additionally, phenylalanine is precursor in synthesis of signalling molecules such as the phytohormone salicylic acid pivotal in launching of plant defence (alternative routes for the synthesis of the hormone exist). Thus, the decrease in levels of amino acids and increase in the levels of some of the downstream derivatives (see **sections 4.4.3** and **4.4.4**) following bacterial treatment, suggests the channelling of these precursors into metabolic pathways of phenylalanine-derived (*e.g.* phenylpropanoids, flavonoids and salicylic acid), tryptophan-derived (*e.g.* indole and serotonin derivatives) and tyrosine-derived defence-related metabolites (Lattanzio *et al.*, 2006; Ishihara *et al.*, 2008; Tzin & Galili, 2010).

4.4.2. Differential changes in fatty acids metabolism following *Burkholderia andropogonis* infection.

The results reveal the significant accumulation of fatty acids in *B. andropogonis*-challenged plants (**Table 4.2**). The levels of fatty acids such as 15-hydroxylinoleic acid, 10,16-dihydroxypalmitate, dihydroxy-octadecadienoic acid, 11,12,13-trihydroxy-9,15-octadecadienoic acid and 9,12,13-trihydroxy-10-octadecenoic acid were found to considerably increase over time (**Figure 4.10**) in the two cvs. However, the accumulation was mostly amplified in the NS 5655 (ST) cv. 10,16-Dihydroxypalmitate, as a practical example, accumulated 24.4-fold in NS 5655 on 7 d.p.i. as compared to 15.6-fold in NS 5511 (**Figure 4.10**). The increase in free fatty acid levels (particularly unsaturated) has been reported in pathogen-stressed plants – palmitoleic acid (16:1) has been linked to increased resistance towards *Verticillium dahlia* in eggplant (Walley *et al.*, 2013; Lim *et al.*, 2017), linoleic (18:2) and linolenic acids (18:3) to *C. gloeosporioides* in avocado and *Pseudomonas syringae* in tomato (Lim *et al.*, 2017). Mutant *Arabidopsis* plants compromised in production of a subclass of fatty acids, (trienoic acids) has been shown to be susceptible to *P. syringae* (Yaeno *et al.*, 2004; Walley *et al.*, 2013).

Compelling evidence from several studies have demonstrated induced activation of NADPH oxidase by linoleic and linolenic acid, leading to production of ROS (Yaeno *et al.*, 2004; Kachroo & Kachroo, 2009; Walley *et al.*, 2013; Lim *et al.*, 2017). The accumulation of ROS can result in the fragmentation/cleavage of fatty acids into various products which can act as chemical inducers of defence responses. In *Arabidopsis* as well as other plants, azelaic acid (a cleavage product) acted as an inducer of SAR *via* the accumulation of SA (Göbel *et al.*, 2002; Walley *et al.*, 2013; Okazaki & Saito, 2014; Gao *et al.*, 2015; Lim *et al.*, 2017). Additionally cell death-inducing activity of some fatty acids has been reported (Göbel *et al.*, 2002).

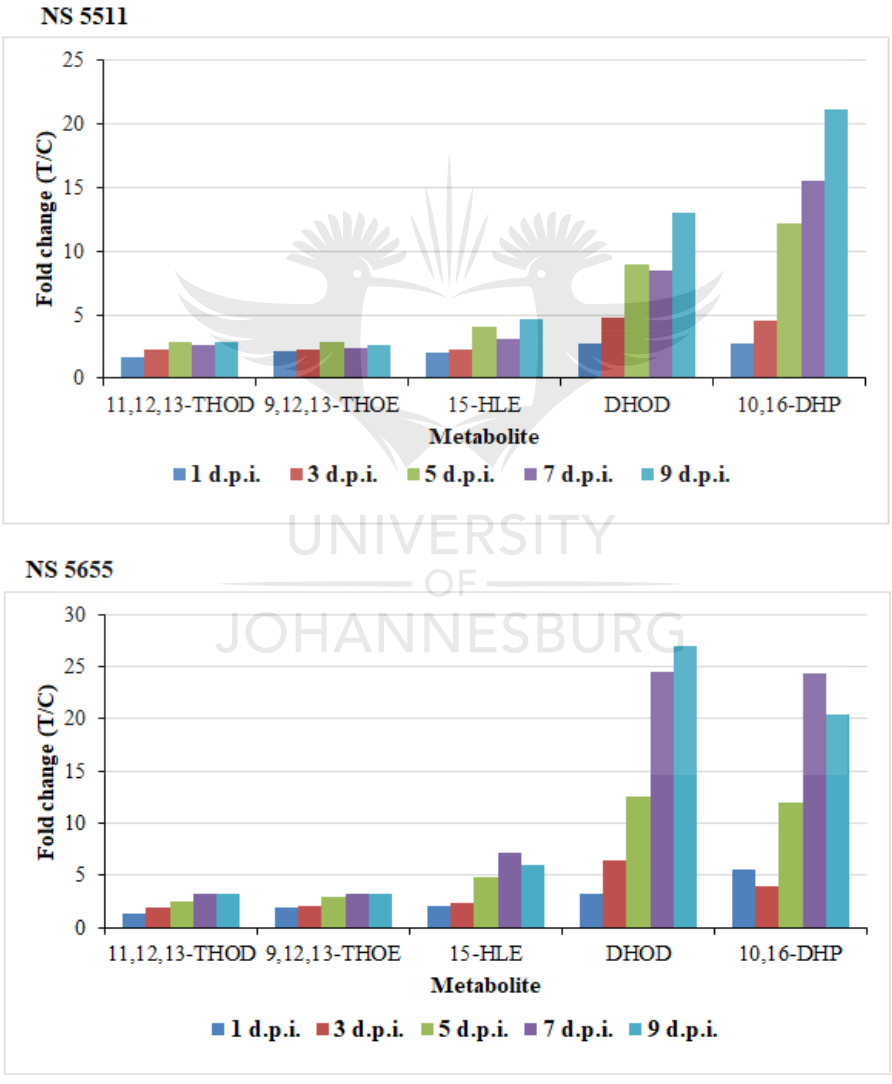

Figure 4.10: Relative quantification of fatty acids annotated in sorghum leaves responding to infection by *B. andropogonis*. The relative levels of each metabolite are expressed in fold changes, computed from treated against control (T/C) where fold change > 1 represents significant accumulation in NS 5511 (BT) and NS 5655 (ST). 11,12,13-THOD = 11,12,13-trihydroxy-9,15-octadecadienoic acid; 9,12,13-THOE = 9,12,13-trihydroxy-10-octadecenoic acid; 15-HLE = 15-hydroxylinoleic acid; DHOD = dihydroxy-octadecadienoic acid; 10,16-DHP = 10,16-dihydroxypalmitate.

The hydroxy fatty acids, 15-hydroxylinoleic acid (avenoleic acid) and 9,12,13-trihydroxy-10-octadecenoic acid (pinellic acid) annotated in this study, have been classified as oxylipins (oxidised fatty acids), synthesised from linoleic acid in cereal crops such as oats and rice (of the Poaceae family) and in other plants (Hamberg & Hamberg, 1996; Hamberg *et al.*, 1998; Aghofack-Nguemezi *et al.*, 2011; Hamberg & Olsson, 2011; Aghofack-Nguemezi & Schwab, 2013). In plants 9,12,13-trihydroxy-10-octadecenoic acid together with other trihydroxyoctadecenoates were reported to be produced in response to fungal infection (conferring resistance to a spectrum of fungal pathogens) and wounding. Previous reports have also highlighted the growth inhibitory roles of trihydroxy derivatives of linoleic and linolenic to plant fungal pathogens (Hamberg, 1997, 1999; Aghofack-Nguemezi *et al.*, 2011; Hamberg & Olsson, 2011). Generally, oxylipins perform defence roles (in plant innate immunity) as signalling molecules – inducing defence responses or as compounds exhibiting antimicrobial properties (Prost, 2005; Eckardt, 2008; Kachroo & Kachroo, 2009; Aghofack-Nguemezi & Schwab, 2013; Walley *et al.*, 2013; Okazaki & Saito, 2014). A study by Prost (2005) demonstrated antimicrobial activities of epoxy- and hydroxy-fatty acids (including others annotated in this study) and other oxylipins towards bacterial pathogens. This study also revealed the significant accumulation of compounds belonging to this class (fatty acids and –derivatives), with reported growth inhibition properties to a spectrum of pathogens.

In local defence, fatty acids (particularly C16 and C18) are involved in the formation of the plant cuticle (comprising of cutin and cuticular wax), a physical barrier limiting nonstomatal water, gas and solutes loss and conferring resistance against bacterial, viral or fungal pathogens. The latter line of defence hinders the pathogen from invading and proliferating in the host plant (Kachroo & Kachroo, 2009; Lim *et al.*, 2017). The synthesis of 10,16-dihydroxypalmitate and 16-hydroxypalmitate (major cutin monomers in flowers, fruits and leaves of plants) annotated in this study might thus be interpreted as an attempt to strengthen the cuticle and limit further bacterial ingress (Li-Beisson *et al.*, 2009; Pushpa *et al.*, 2014; Barbaglia & Hoffmann-Benning, 2016).

Apart from acting as hydrophobic hormones in modulating signal transduction pathways, fatty acids also serve as precursors to the phytohormone jasmonic acid (Prost, 2005; Kachroo & Kachroo, 2009; Hamberg *et al.*, 2003; Walley *et al.*, 2013), and as essential constituents of membrane lipids in plants. The up-regulation of the annotated fatty acids of known function,

thus, hints to their functional role in sorghum defence/resistance (Weber, 2002; Kachroo & Kachroo, 2009; Walley *et al.*, 2013; Lim *et al.*, 2017).

4.4.3 Plant hormones: regulatory and signalling molecules in sorghum defence responses

The intricate defence responses of sorghum also involved several phytohormones. SA and SA glucoside, jasmonic acid and zeatin derivatives were annotated (**Table 4.2**). Drawing attention to the well-known plant stress phytohormone, SA and conjugate thereof (salicylic acid 2-O-beta-D-glucoside (SAG), augmented levels were noticed following the bacterial treatment (**Figure 4.11**). SA levels in NS 5511 (BT) were elevated in the early stages of bacterial infection (1-3 d.p.i.) as compared to NS 5655 (ST), which showed increased levels in the late stages (5-9 d.p.i.). This finding postulates an early onset of defence responses in NS 5511 compared to NS 5655, and corroborates the view that the former is more resistant than the latter. However, for SAG the levels between the two cvs were comparable. As outlined in **Chapter 2**, **section 2.3.2** plant hormones play various biological roles in plants, including signalling in stress responses, inducing secondary metabolites accumulation (Cheynier *et al.*, 2013; Pieterse & Van Wees, 2015; Mhlongo *et al.*, 2016; Wani *et al.*, 2016; Tugizimana, 2017). Experimental evidence has shown a link between the accumulation of SA (and its glucoside), expression of PR proteins, accumulation of phenylpropanoids and resistance to pathogens, in a range of plants (Sudha & Ravishankar, 2002; Grüner *et al.*, 2003; Zeier, 2013; Okazaki & Saito, 2014; Finnegan *et al.*, 2016). SA, a phenolic phytohormone synthesised from isochorismate *via* the shikimic acid pathway (Tzin & Galili, 2010; Zeier, 2013; Lim *et al.*, 2017) plays a key role in local and systemic defence (Sudha & Ravishankar, 2002; Ramirez-Estrada *et al.*, 2016).

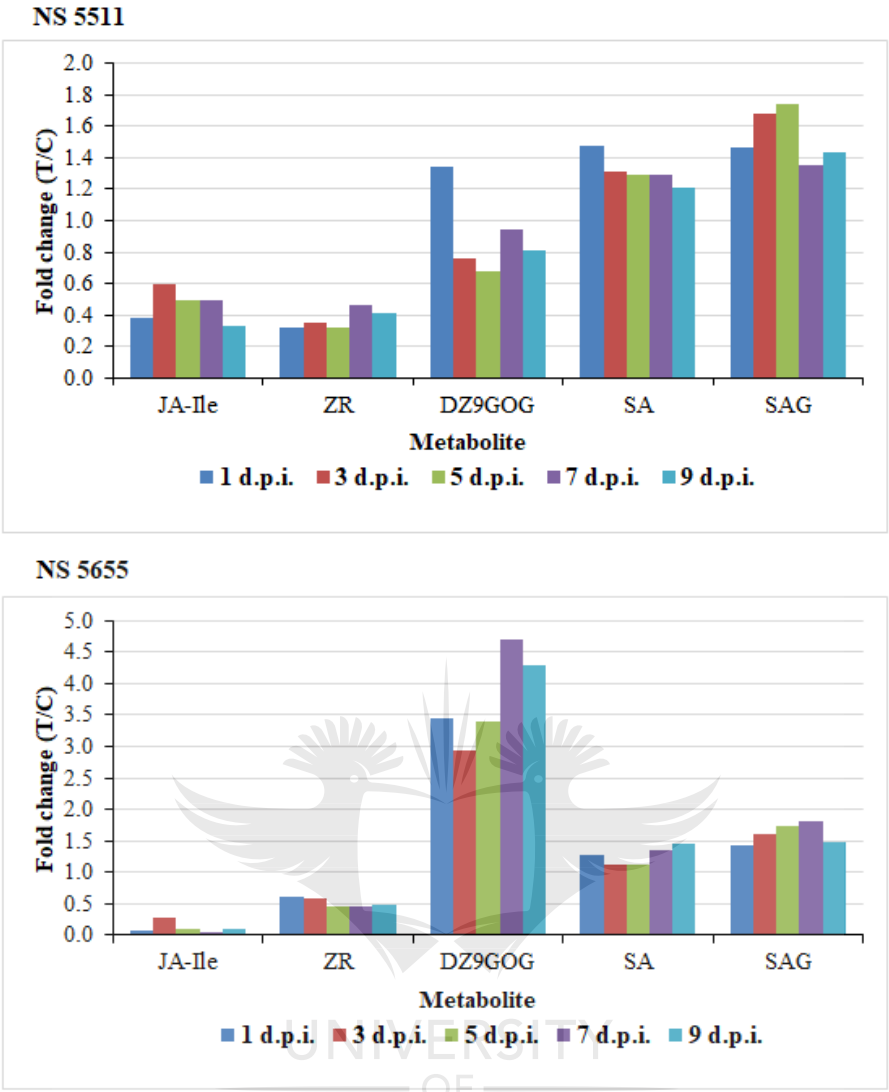

Figure 4.11: Relative quantification of plant hormones annotated in sorghum leaves responding to *B. andropogonis* infection. The relative levels of each metabolite are expressed in fold changes, computed from treated against control (T/C) where fold change > 1 represents significant accumulation in NS 5511 (BT) and NS 5655 (ST). JA-Ile = jasmonoyl-L-isoleucine; ZR = zeatin riboside; DZ9GOG = dihydrozeatin-9-N-glucoside-O-glucoside; SA = salicylic acid; SAG = salicylic acid 2-O-beta-D-glucoside.

SA accumulates in regions around the infection site, stimulating the hypersensitive response (HR) – a type of programmed cell death at the site of infection which results in necrotic lesions and limiting pathogen proliferation. This HR can be linked to the development of lesions observed on *B. andropogonis*-treated sorghum plants (**section 4.1**, **Figure 4.1-2**) (Alvarez, 2000; Grüner *et al.*, 2003; Mur *et al.*, 2008; Kachroo & Kachroo, 2009; Finnegan *et al.*, 2016). Moreover the phytohormone is the major signalling molecule triggering SAR (a systemic form of resistance, arming distal un-infected parts of the plant against subsequent secondary infections), leading to up-regulation of PR proteins and enhancement of phenylpropanoids accumulation (Sudha & Ravishankar, 2002; Bigeard *et al.*, 2015; Cuperlovic-Culf *et al.*, 2016),

and its production and signalling function is highly important in plant immunity towards pathogens exhibiting biotrophic and hemibiotrophic lifestyles. The detected levels of SA and SAG, therefore suggests that *B. andropogonis* treatment triggered SAR (Mengiste, 2012; Zeier, 2013; Andolfo & Ercolano, 2015; Bigeard *et al.*, 2015; Cuperlovic-Culf *et al.*, 2016).

The derivative of the phytohormone JA, jasmonoyl-L-isoleucine, was also amongst the annotated metabolites. The conjugation of jasmonate and isoleucine results in the highly biologically active jasmonoyl-L-isoleucine which plays a role in defence responses in the event of stress (Suza *et al.*, 2010; Koo *et al.*, 2011; Woldemariam *et al.*, 2012; Liu *et al.*, 2015; Barbaglia & Hoffmann-Benning, 2016; Ishimaru *et al.*, 2017; Koo, 2017). Although this phytohormone was identified to be present, it was not statistically significant and was thus not picked to be significantly related to the treatment. This is indicated by the very low levels detected upon quantitative evaluation (**Table 4.2** and **Figure 4.11**). Jasmonates (derived from fatty acid metabolism; linolenic acid) are also classified as oxylipins (see **section 4.4.2** on literature regarding oxylipins) widely spread in the plant kingdom. Apart from being involved in developmental processes, these molecules are also involved in activation of defence responses towards abiotic and biotic stressors (directly or indirectly), as signalling molecules (Sudha & Ravishankar, 2002; Kachroo & Kachroo, 2009; Okazaki & Saito, 2014; Wani *et al.*, 2016; Lim *et al.*, 2017). Exogenous application of JA results in defence-related gene expression as well as production of antimicrobial compounds (Sudha & Ravishankar, 2002; Kachroo & Kachroo, 2009; Mhlongo *et al.*, 2016). The accumulation of this phytohormone is mostly associated with necrotrophic pathogens and also triggers ISR (Kachroo & Kachroo, 2009; Antico *et al.*, 2012; Pieterse & Van Wees, 2015; Mhlongo *et al.*, 2016; Wielkopolan & Obrępalska-Stęplowska, 2016).

The levels of two zeatin conjugates, dihydrozeatin-9-N-glucoside-O-glucoside and zeatin riboside, were also found to be altered (with the former being significantly altered) as part of induced host responses following bacterial treatment. Dihydrozeatin-9-N-glucoside-O-glucoside levels demonstrated to generally decrease over time in NS 5511 while increasing in NS 5655 (**Figure 4.11**). Zeatin and the derivatives thereof (*e.g.* riboside and glucosides) are regarded as the principal group of isoprenoid cytokinins in plants. Previously *trans*-zeatin cytokinins were demonstrated to be more active in enhancing resistance against pathogens, however *cis*-zeatin CKs have recently been reported in regulating plant defence responses in pathogen challenge and the potential role as 'novel' stress-response markers has been

highlighted (Großkinsky *et al.*, 2013; Schäfer *et al.*, 2015). Cytokinins have been shown to act synergistically with SA in the activation of defence gene expression. Recently in *Arabidopsis*, the class of hormones was demonstrated to regulate SA signalling pathways and enhance resistance to *P. syringae* pv. *tomato* DC3000 and *Hyaloperonospora arabidopsidis* exhibiting hemibiotrophic and biotrophic lifestyles, respectively (Jiang *et al.*, 2013). In *Arabidopsis* and tobacco, enhancing of resistance by zeatin cytokinins was demonstrated to be linked to increase in cell membrane integrity (Schäfer *et al.*, 2015; Shigenaga & Argueso, 2016).

The identification of the various hormones revealed an interplay of plant hormones in sorghum defence signalling and regulation. Interaction (antagonistically or synergistically) and fine tuning between plant hormones governs activation of a range of defences including those specific to the stressor (Kachroo & Kachroo, 2009; Antico *et al.*, 2012; Jiang *et al.*, 2013; Tugizimana *et al.*, 2014; Burketova *et al.*, 2015; Wani *et al.*, 2016). Alterations in hormone levels revealed by quantitative analysis can therefore be linked to triggering of defence responses to *B. andropogonis* infection.

4.4.4. Metabolic reprogramming of defence-related metabolites derived from shikimic acid-, phenylpropanoid-, and flavonoid pathways

The metabolic reprogramming in sorghum following *B. andropogonis* infection, involved perturbations in the pool of metabolites synthesised *via* the shikimic acid, phenylpropanoid, and flavonoid biosynthetic pathways – which are partially interlinked (Lo & Nicholson, 1998; Dixon *et al.*, 2002; Tugizimana, 2017). The shikimic acid pathway yields chorismic acid – a precursor in the aromatic amino acid biosynthetic pathways. These two pathways portray a prime regulatory link of primary and secondary metabolism. Phenylalanine serves as an initiator/regulatory metabolite in the biosynthesis of phenylpropanoids (of which flavonoid pathway is a downstream branch) (Dewick, 2002; Tzin & Galili, 2010; Tohge *et al.*, 2013; Mhlongo *et al.*, 2014, 2016; Mierziak *et al.*, 2014; Ng *et al.*, 2016).

Quantitative analysis revealed a decrease in chorismic acid levels in the NS 5655 cv which was, however, not annotated in the NS 5511 cv (maybe due to levels being quite low to be detected) (**Table 4.2**). As stated previously, chorismic acid provides a carbon skeleton in the synthesis of aromatic acids, from which aromatic secondary metabolites arise (Tzin & Galili, 2010; Tohge *et al.*, 2013; Shigenaga & Argueso, 2016). Benzoic acid and the 3,4,5-trihydroxy-

derivative (gallic acid monohydrate) were also detected, with the levels of the latter significantly higher compared to the former in both cvs. Benzoic acid and derivatives are known for their antioxidant and antimicrobial activity. The decrease in levels of chorismic and benzoic acid can be attributed to channelling of the metabolites into synthesis of various defence-related metabolites to which the metabolites serve as precursors (Fogliani *et al.*, 2005; Karamac *et al.*, 2006; Zhao *et al.*, 2011).

Two major groups of phenolic compounds derived from the phenylpropanoid pathway and regarded as the major phenolic compounds found in sorghum – flavonoids and hydroxycinnamic acids, largely accumulated in treated plants. The profusion of these classes of compounds amongst the putatively identified metabolites (**Figure 4.9 A** and **Table 4.2**) suggests a pivotal role in sorghum defence (as either preformed phytoanticipins or induced phytoalexins) (Awika & Rooney, 2004; Taylor *et al.*, 2014; Tugizimana *et al.*, 2014; Kang *et al.*, 2016). Phenolic secondary metabolites are a major group of secondary metabolites directly involved in plant resistance and in determining resistance/susceptibility of a plant host to microbial pathogens (Bollina *et al.*, 2010; Tzin & Galili, 2010; Anjum *et al.*, 2013; Tohge *et al.*, 2013; Liu *et al.*, 2016). Many reports have demonstrated accumulation of phenolic compounds at the site of infection following pathogen invasion (Bollina *et al.*, 2010; Mierziak *et al.*, 2014).

4.4.4.1. Flavonoids as biomarkers in sorghum defence responses against *Burkholderia andropogonis*

The flavonoids annotated from the treated plants extracts (**Table 4.2**) were mostly sugar-conjugated and belonged to various subgroups; (i) **flavones** – apigenin and derivatives luteolin and tricetin derivatives, (ii) **flavanones** – naringenin derivatives, (iii) **flavonols** – quercetin derivatives and (iv) **chalcones** – hydroxychalcone derivatives. A schematic representation of the proposed flavonoid biosynthetic pathway leading to the synthesis of some flavonoids annotated in this study (indicated in bold), as defence-related metabolites, is infographically shown in **Figure 4.12**. The various flavonoid subgroups to which the defence-related metabolites belong to are colour coded (see key on the top left of **Figure 4.12**).

Interestingly, most of the detected flavonoid glycosides significantly accumulated following bacterial infection. Flavonoids are a highly diverse class of secondary metabolites (Martens &

Mitho, 2005; Abad-Garcı´a *et al.*, 2008; Bollina *et al.*, 2010), with a wide range of biological functions in the plant system which include signalling, abiotic and biotic stress response, and antioxidant activity, amongst others. Synthesis, transportation and allocation of this class of compounds hallmarks an adaptive metabolism in plants (in protective and regulatory functions) (Winkel, 2004; Devi *et al.*, 2011; Du Fall & Solomon, 2011; Falcone Ferreyra *et al.*, 2012; Petrussa *et al.*, 2013; Mierziak *et al.*, 2014). Upon pathogen challenge, flavonoids accumulate at the infection site and impede fungal spore germination, inactivate bacterial pathogen adhesion and distort microbial membranes (amongst other mechanisms), all in attempt to hinder microbial invasion (Mishra *et al.*, 2009; Naoumkina *et al.*, 2010; Du Fall & Solomon, 2011; Mierziak *et al.*, 2014).

The results showed that the metabolic changes in flavonoid metabolism following bacterial infection were largely characterised by a significant accumulation of apigenin and its glycosides (mostly existing as *C*-glycosides) (**Table 4.2**). The aglycone apigenin displayed an increase in levels over time points particularly from 3-9 d.p.i. (levels ≥ 1.5-fold) (**Figure 4.13**). For NS 5655, levels of this metabolite seemed a bit higher than in NS 5511. On the other hand, apigenin glycosides (found to be constitutively present in sorghum plants this study *i.e.* as phytoanticipins) levels appeared to be more augmented in the latter compared to the former. Rhoifolin (apigenin-7-O-neohesperidoside) for instance, displayed its highest level at 7 d.p.i. as 1.9-fold in NS 5511 and 1.4-fold in NS 5655, respectively, as infographically shown in **Figure 4.13**.

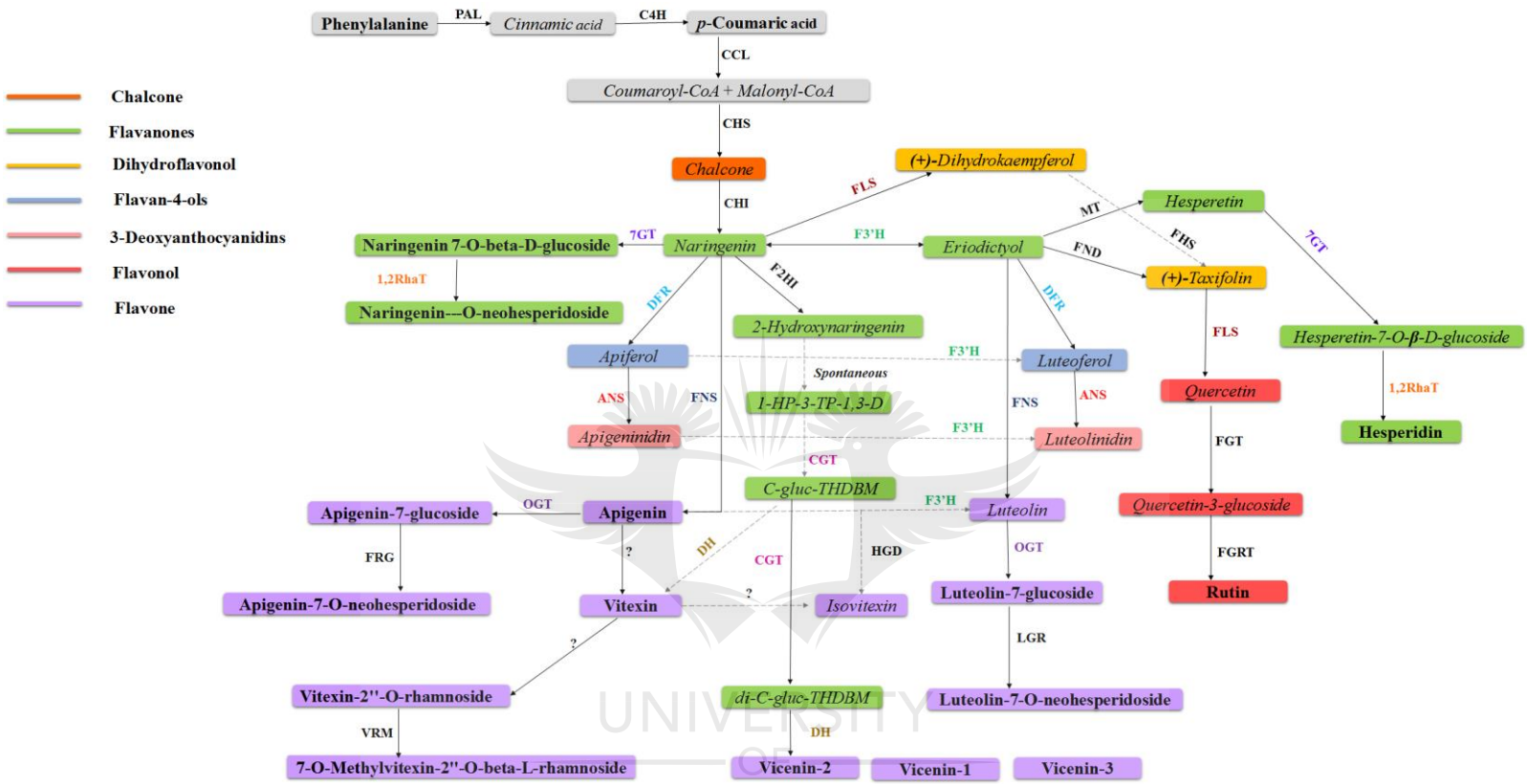

Figure 4.12: Schematic representation of proposed biosynthetic pathway of defence-related flavonoids in sorghum. This pathway displays some of the defence-related flavonoids annotated in sorghum plant extracts following treatment with *B. andropogonis*. Solid lines represent main routes and dashed lines represent alternative routes. Abbreviations: PAL = phenylalanine ammonia lyase; C4H = cinnamate 4-hydroxylase; CCL = coumaryl-CoA ligase; CHS = chalcone synthase; CHI = chalcone isomerase; F3'H = flavonoid 3'-hydroxylase; 7GT = flavanones-7-*O*-glucosyltransferase; Cm1,2RhaT = 1,2 rhamnosyltransferase; DFR = dihydroflavonol reductase; ANS = anthocyanidin synthase; F2HI = flavanone-2-hydrohylase; FNS = flavone synthase; OGT = *O*-glycosyltransferase; FRG = flavanones 7-O-glucoside-2"-*O*-β-L-rhamnosyltransferase; LGR = luteolin 2-*O*-β-L-rhamnosyltransferase; VRM = vitexin 2"-*O*-rhamnoside 7-*O*-methyltransferase; MT = methyltransferase; FND = flavanones 3-dioxygenase; FLS = flavonol synthase; FGT = flavonoid 3-*O*-glucosyltransferase; FGRT = flavonol-3-*O*-glucoside L-rhamnosyltransferase; FHS = flavonoid 3',5'-hydroxylase; CGT = *C*-glycosyl transferase; HGD = 2-hydroxynaringenin-6-*C*-glucoside dehydratase; DH = dehydrogenase; ? = enzymes not yet characterised. Metabolites annotated in this study = in bold; metabolites not annotated in this study = italicised. Flavonoid classes are colour-coded as shown in the key (top left) (adapted from Boddu *et al.*, 2004; Frydman *et al.*, 2004; Casas *et al.*, 2014; Poloni & Schirawski, 2014; https://MetaCyc.org; Abdullah & Chua, 2017).

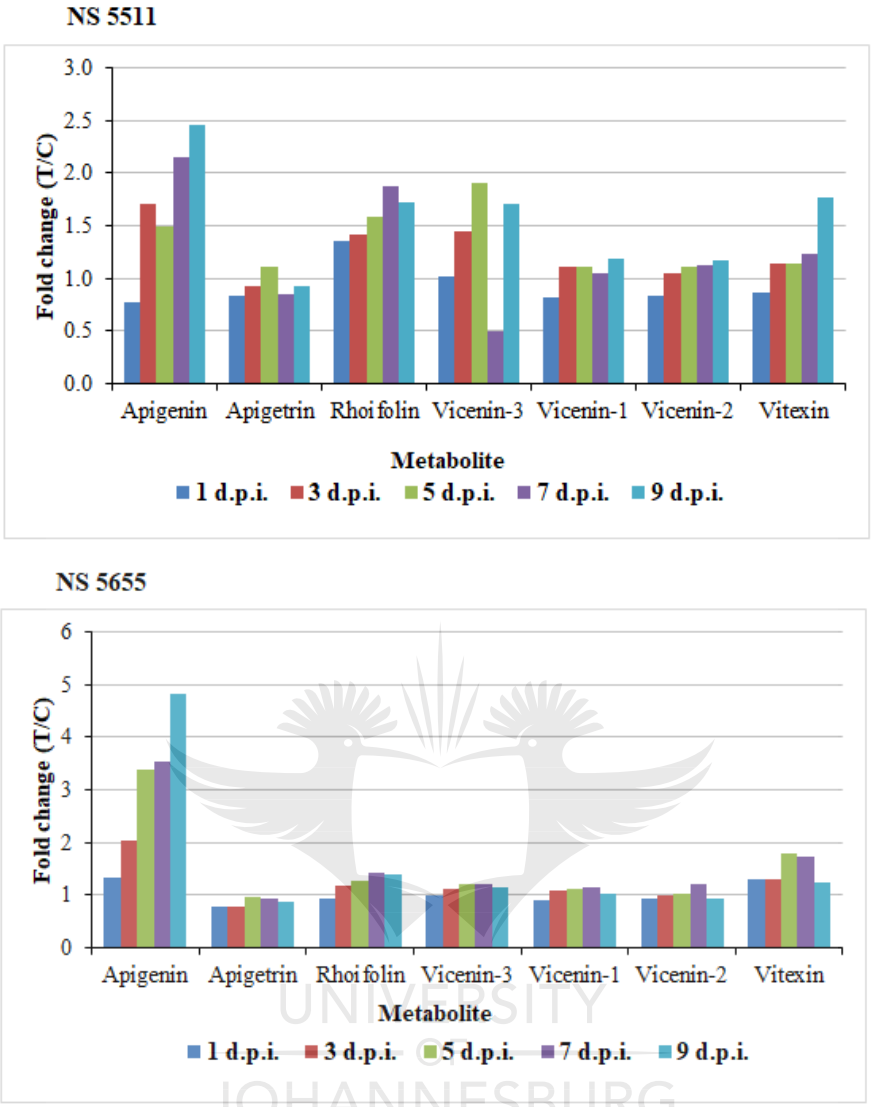

Figure 4.13: Relative quantification of apigenin and conjugates annotated in sorghum leaves responding to *B. andropogonis* infection. The relative levels of each metabolite are expressed in fold changes, computed from treated against control (T/C) where fold change > 1 represents significant accumulation in NS 5511 (BT) and NS 5655 (ST). Apigetrin = apigenin 7-O-glucoside; rhoifolin = apigenin 7-O-neohesperidoside; vicenin-3 = apigenin 8-C-xyloside-6-C-glucoside; vicenin-1 = apigenin 6-C-xyloside-8-C-glucoside; vicenin-2 = apigenin-6,8-di-C-glucoside; vitexin = apigenin-8-*C*-glucoside.

In this study, apigenin demonstrated to be pathogen-induced bio-marker in both cvs. This finding was visually confirmed by exploration of the PCA scores space. The metabolite was not detected in the non-treated (control) samples but was clearly detectable in the treated samples, with levels increasing over time and most intensely in NS 5655 (**Figure 4.14**). Apigenin is a well-known phytoalexin in sorghum, contributing to host resistance. Studies have shown *de novo* rapid and elevated accumulation of the metabolite in sorghum following infection (Du *et al.*, 2010; Du Fall & Solomon, 2011; Ahuja *et al.*, 2012; Mizuno *et al.*, 2016;

Schnippenkoetter *et al.*, 2017). The flavone has been demonstrated to inhibit fungal growth and spore germination against fungal pathogens such as *Colletotrichum sublineolum* (Du Fall & Solomon, 2011; Ahuja *et al.*, 2012). For bacterial pathogens, apigenin has been shown to exhibit a stronger antibacterial activity towards Gram-negative bacteria (Basile *et al.*, 1999).

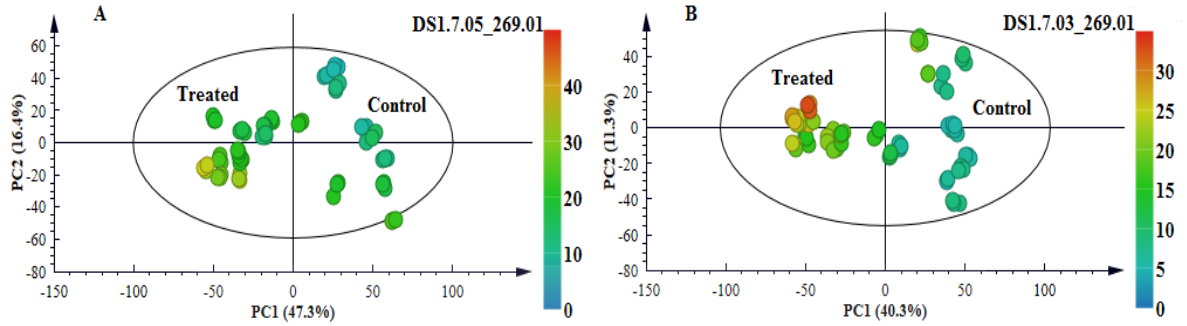

Figure 4.14: An unsupervised colour-coded PCA score plot displaying the presence/absence and intensity of the phytoalexin apigenin across the samples. (A): NS 5511 (BT) and **(B):** NS 5655 (ST). The absence of the metabolite in non-treated (control) samples and presence in the treated samples indicates that the metabolite was pathogen-induced.

The apigenin conjugates, rhoifolin and vitexin, found to significantly accumulate in treated samples (**Figure 4.13**), have also been reported to possess antimicrobial properties. Rhoifolin was demonstrated to exhibit antimicrobial activity to the bacterial pathogen *Escherichia coli* (Tian *et al.*, 2009; Refaat *et al.*, 2015). Vitexin, on the other hand, was shown to confer resistance in plants such as barley (towards *Fusarium graminearum*) (Balmer *et al.*, 2013), cucumber (towards *Podosphaera xanthii* and other fungal pathogens) (Mohamed & El-hadidy, 2008; Du *et al.*, 2010) and *Linum usitatissimum* (towards *F. oxysporum* and *F. culmorum*) (Mierziak *et al.*, 2014).

Vitexin (and related conjugates), and the other apigenin glycosides vicenin-1, vicenin-2 and vicenin-3, were reported for the first time in sorghum, in line with defence responses. In addition to vicenin-2 exhibiting antioxidant activity in plant defence (against abiotic stress such as UV damage) (Silva *et al.*, 2014; Spínola *et al.*, 2015), *in vitro* and *in vivo* studies demonstrated the antifungal activity of vicenin-2 together with other phenolic compounds extracted from *Verbascum eremobium* towards a range of fungal pathogens in cucumber (Mohamed & El-hadidy, 2008). The levels of vicenin-1, vicenin-3 and the apigetrin (**Figure 4.13**) in treated plants suggests a defence-related role towards *B. andropogonis*, however,

further work is required to elucidate and explore the particular roles in sorghum-pathogen defence.

Luteolin, another known phytoalexin of sorghum was not detected in this study, however, two of its conjugates luteolin 7-O-glucoside and luteolin 7-O-neohesperidoside were annotated. These two metabolites accumulated significantly in the NS 5511 cv as compared to NS 5655 cv (**Table 4.2**), with levels appearing to increase over time (data not shown). Luteolin 7-O-neohesperidoside has been reported as an antibacterial compound while luteolin 7-O-glucoside has been shown to possess antifungal activity contributing to host resistance (Basile *et al.*, 1999; Mohamed & El-hadidy, 2008; Tian *et al.*, 2009).

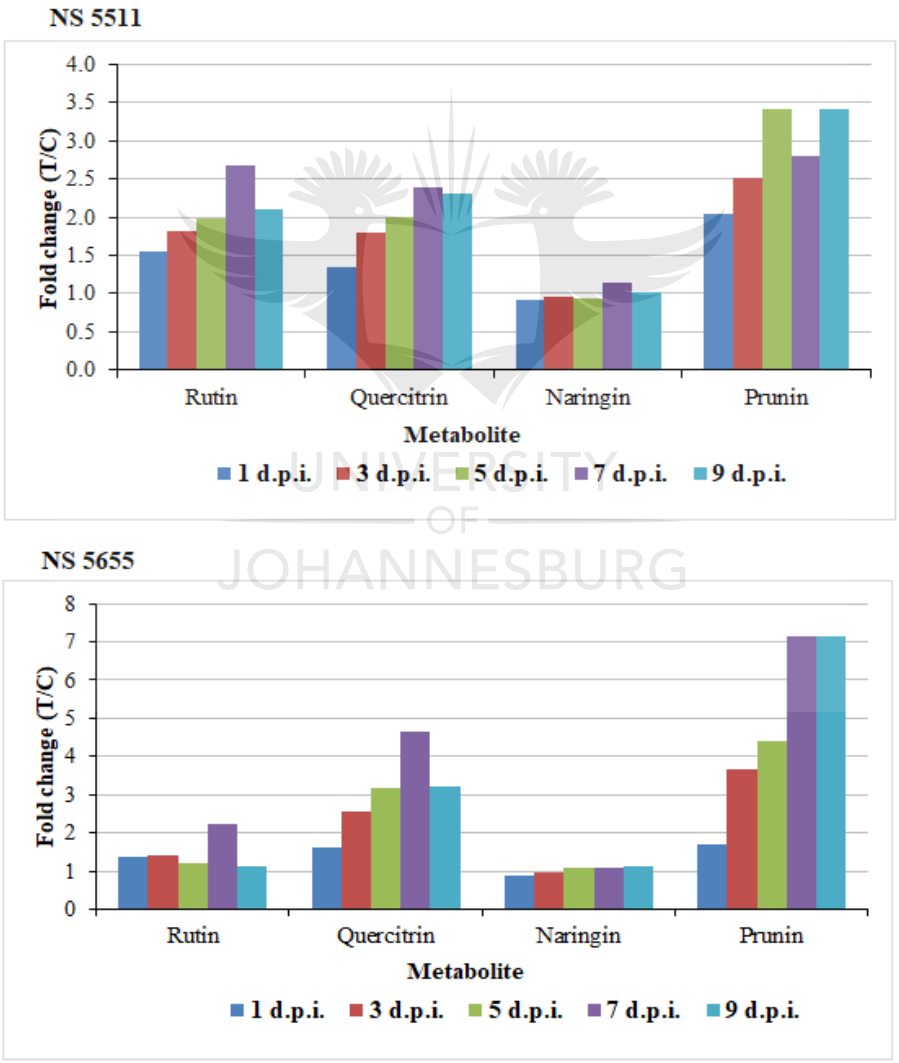

Figure 4.15: Relative quantification of flavanones and flavonols annotated in sorghum leaves responding to *B. andropogonis* infection. The relative levels of each metabolite are expressed in fold changes, computed from treated against control (T/C) where fold change > 1 represents significant accumulation in NS 5511 (BT) and NS 5655 (ST). Rutin = quercetin rutinoside; quercitrin = quercetin 3-O-rhamnoside; naringin = naringenin 7-O-neohesperidoside; prunin = naringenin 7-O-beta-D-glucoside.

Other flavonoids found to be up-regulated following bacterial infection (**Table 4.2** and **Figure 4.15**), but more significantly in NS 5655, are naringenin, quercetin and chalcone conjugates. These conjugates have been linked to defence in plants following pathogen challenge. Naringenin 7-O-beta-D-glucoside and naringin have been shown to exhibit antimicrobial activity against *F. graminearum* in wheat (Gunnaiah & Kushalappa, 2014; Mierziak *et al.*, 2014) and barley (Chamarthi *et al.*, 2014), and *Penicillium digitatum* in *Citrus aurantium* (Arcas *et al.*, 2000; Ortuño *et al.*, 2006; Treutter, 2006) respectively. The former was also shown to enhance resistance in barley against the pathogen *Gibberella zeae* (Bollina *et al.*, 2010; Gunnaiah & Kushalappa, 2014; Mierziak *et al.*, 2014). Chalcones were demonstrated to exhibit antibacterial activity towards *Cladosporium cucumerinum* in *Mariscus psilostachys*, and quercetin derivatives exhibited antifungal activity (inhibiting spore germination) (Lattanzio *et al.*, 2006; Abdel-Farid *et al.*, 2009; Mierziak *et al.*, 2014).

Monitoring the metabolic changes in *B. andropogonis*-challenged sorghum plants revealed accumulation of flavones, flavanones, flavanol and chalcones, but not the rare class of anthocyanidins, 3-deoxyanthocyanidins, previously reported for fungal infections. As the colour of the host lesions typically depends on the affected plant's response (Claflin *et al.*, 1992), Mizuno *et al.* (2016) described sorghum plants with a tan pigmentation response, as lacking apiferol and luteoferol, precursors of apigeninidin and luteolinidin, respectively (see **Chapter 2**, **Figure 2.8**), thus, the inability to synthesise this class of 3-deoxyanthocyanidins. Sorghums with purple or red pigmentation, were however, described as accumulating 3-deoxyanthocyanidins. On the other hand, sorghum plants with tan pigmentation in response to stress (as also observed following symptom evaluation in **see section 4.1**), were reported as associated with high accumulation of apigenin and luteolin (similar to response observed this study particularly for the former *i.e.* apigenin and glycosides). The above can explain why the rare class of anthocyanidins were not annotated in our extracts – thus we can suggest that *B. andropogonis* infection did not activate this anthocyanidin biosynthetic pathway (Boddu *et al.*, 2004; Basavaraju *et al.*, 2009; Poloni & Schirawski, 2014; Meyer *et al.*, 2015; Mizuno *et al.*, 2016). We can, therefore, postulate that defence responses launched towards *B. andropogonis* infection by sorghum largely involves the subgroups of flavonoids mentioned above, particularly flavones.

4.4.4.2. The defensive functions of hydroxycinnamic acids in sorghum

Treated sorghum plants accumulated hydroxycinnamic acids in response to *B. andropogonis*. The annotated hydroxycinnamic acids included coumaric acid, caffeic acid, sinapic acid, coniferyl and ferulic acid conjugated to various molecules such as sugars, organic acids, alcohols, aldehydes and amines **(Table 4.2).** As displayed in the **Table 4.2**, these compounds accumulated in varying degrees in the two cvs. Sinapoyl alcohol, 4-coumaroylquinic acid, 3-feruloylquinic acid, 1-O-coumaroyl-beta-D-glucose, and 1,2-bis-O-sinapoyl-beta-D-glucoside, were up-regulated (**Table 4.2**) following bacterial treatment. Furthermore, relative quantification analysis (**Figure 4.16)** generally showed an increase in relative levels over time. Comparison of the two cvs displayed a more significant accumulation in NS 5511. *p*-Coumaric acid, ferulic acid and 4-coumaroylagmatine (also shown in **Figure 4.16**), however, display a decrease in levels across time.

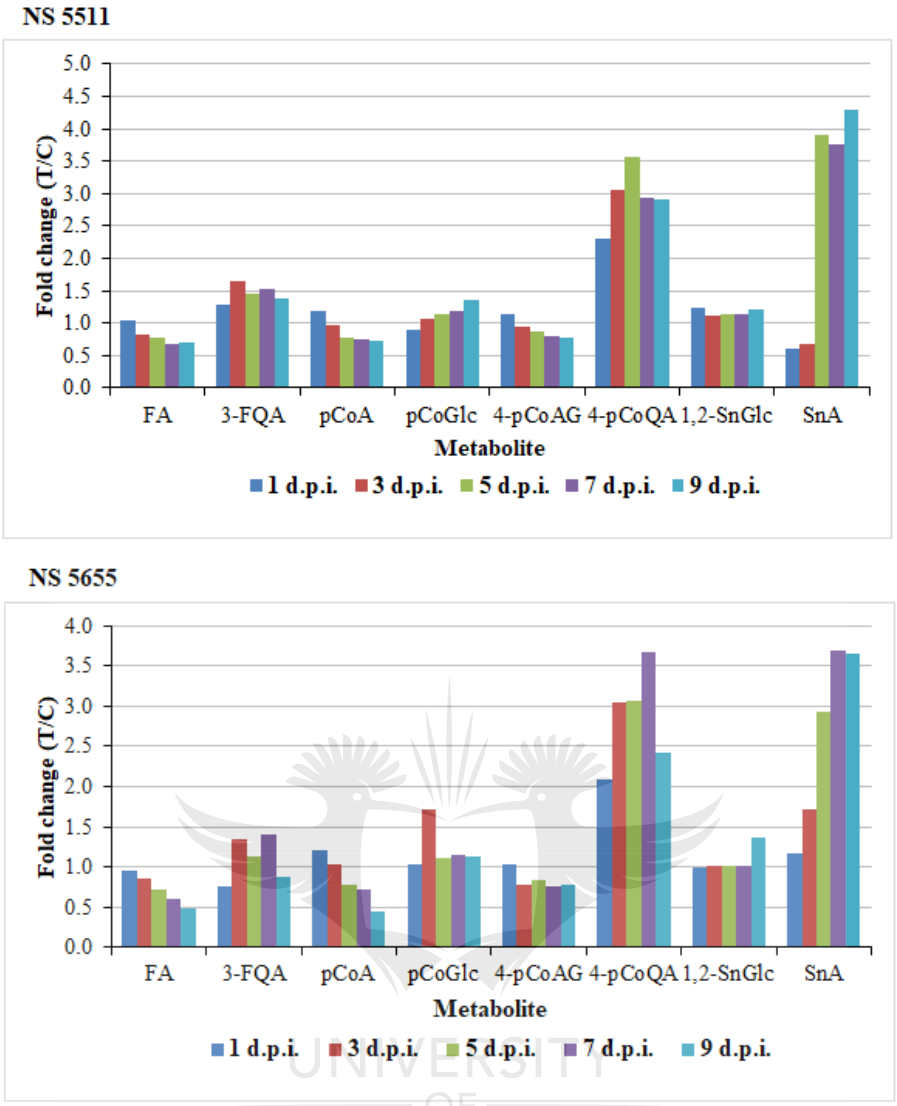

Figure 4.16: Relative quantification of hydroxycinnamic acids annotated in sorghum leaves responding to *B. andropogonis* infection. The relative levels of each metabolite are expressed in fold changes, computed from treated against control (T/C) where fold change > 1 represents significant accumulation in NS 5511 (BIT) and NS 5655 (ST). FA = ferulic acid; 3-FQA = 3-feruloylquinic acid; *p*CoA = *p*-coumaric acid; *p*CoGlc = 1-O-coumaroyl-beta-D-glucose; 4-*p*CoAg = 4-coumaroylagmatine; 4-pCoQA = 4-coumaroylquinic acid; 1,2-SnGlc =1,2-bis-O-sinapoyl-beta-D-glucoside; SnA = sinapoyl alcohol.

Phenylpropanoids including hydroxycinnamic acids are known to possess defence-related functions. Ferulic-, caffeic-, *p*-coumaric- and sinapic acids are functional antimicrobial compounds and precursors to the synthesis of inducible (phytoalexins) and constitutive (phytoanticipins) defence metabolites. The metabolites are also key in structural defences by participating in cross linking primary cell wall polysaccharides in addition to being lignin precursors (Dixon *et al.*, 2002; Bollina *et al.*, 2010; Gunnaiah & Kushalappa, 2014; Mhlongo *et al.*, 2014; Tugizimana *et al.*, 2014; Lowe *et al.*, 2015).

Ferulic acid and other cinnamic acids were shown to inhibit *F. graminearum* progression and production of mycotoxins by this pathogen (Bollina *et al.*, 2010; Gunnaiah & Kushalappa, 2014; Yogendra *et al.*, 2014). Hydroxycinnamic acid amides such as 4-coumaroylagmatine and feruloylserotonin, are known in the context of plant defence; strengthening/thickening cell walls and as antimicrobial compounds. A study on potato cultivars showed the accumulation of these hydroxycinnamic acid amides and other hydroxycinnamic acids; 4-coumaroylquinic acid, feruloylquinic acid, 1-O-sinapoyl-beta-D-glucose, 4-hydroxycoumarin, 1-O-feruloyl-beta-D-glucose in resistant cultivars in response to *Phytophthora infestans* (Gunnaiah & Kushalappa, 2014; Pushpa *et al.*, 2014; Yogendra *et al.*, 2014; Macoy *et al.*, 2015). Similarly, a number of these metabolites reported in potato and also detected in this study were associated with pathogen resistance in some members of the Poaceae family such as wheat and barley (Bollina *et al.*, 2010; Gunnaiah *et al.*, 2012; Gunnaiah & Kushalappa, 2014; Cuperlovic-Culf *et al.*, 2016). The observed significant accumulation of these hydroxycinnamic acids (as stated in the beginning of this section), can thus, be linked to defence-related functions in sorghum towards *B. andropogonis*.

Sinapoyl alcohol derived from cinnamic acid *via* p-coumaric-, caffeic-, ferulic- and sinapic acid intermediates, is an important precursor (together with sinapaldehyde also annotated in this study) in plant cell wall lignification – a structural defence mechanism to hinder pathogen penetration. The decrease in levels of 1-O-sinapoyl-beta-D-glucose (not shown) accompanied by higher levels of its active form, sinapoyl alcohol (**Figure 4.16**), displayed a conversion of an inactive to an active form which marks the activation of structural defences (to strengthen the cell wall). The sinapoyl glucoside and 1-O-feruloyl-beta-D-glucose are inactive storage forms which are activated due to pathogen infection to form sinapoyl alcohol and feruloyl alcohol respectively (the latter – not detected in this study) (Vanholme *et al.*, 2010; Pushpa *et al.*, 2014; Yogendra *et al.*, 2014). Sinapoyl alcohol increased more significantly in the NS 551 cv. Other metabolites also involved in structural defence are coniferyl acetate, sinapaldehyde glucoside and sinapoyl-(S)-malate. The significant accumulation of hydroxycinnamic acid conjugates associated with a decrease in the precursors, therefore, shows channelling of the latter into synthesis of corresponding derivatives more important for sorghum resistance (Bollina *et al.*, 2010; Gunnaiah & Kushalappa, 2014; Gauthier *et al.*, 2015; Cuperlovic-Culf *et al.*, 2016; Le Roy *et al.*, 2016; Sun *et al.*, 2016).

Table 4.2 also indicates metabolites (detected in both ESI positive and negative ionisation modes) that were not annotated *i.e.* not assigned putative names. The possible empirical formulae computed based on accurate mass, obtained from the Waters SYNAPT G1 Q-TOF system, was assigned to the unknowns (as described in the experimental section in **Chapter 3**). However, these ions/metabolites were identified as very significant discriminant biomarkers by the MVDA, and identification may be possible in the future.

The changes observed in metabolite levels of the NS 5511 and NS 5655 cvs (increases or decreases) over time, following relative quantitative analyses, explain the differences visualised in chromatographic analyses (BPI LC-MS chromatograms; **section 4.2**) as well as the clustering patterns of samples in the PCA scores plots (**section 4.3**) *i.e.* time-related changes/differences. Moreover, the presence/absence of metabolites and the varying degrees in metabolite accumulation in the two *S. bicolor* cvs, point to underlying differential metabolism between the two. These results suggest that both cvs respond to pathogen attack (as described in the section on innate immunity in **Chapter 2**), but that the 'defensomes' to *B. andropogonis* differ due to differential metabolic reprogramming, thus contributing and explaining their differences in the resistant/tolerant/susceptible phenotypes. The observed nuances can be attributed to genetic factors and factors controlling the kinetics of the induced defence responses and the extent to which activation occurs (Woodhead, 1981; Wu *et al.*, 2016; Tugizimana, 2017).

Based on the disease severity-rating index (**section 4.1**), the NS 5511 (BT) cv demonstrated to be more resistant compared to the NS 5655 (ST) cv, as it symptomatically displayed delayed signs of wilting and spreading of lesion compared to the latter. This observation corroborates with Tugizimana (2017), and is supported by the early accumulation (1-5 d.p.i.) of the phytohormone salicylic acid in significant levels, in the NS 5511 cv – important in orchestrating defence responses and systemic resistance. In the NS 5655 cv the levels of the hormone appeared to increase only at later stages of the treatment. The above observation suggests that timing and intensity of the accumulation of crucial defence metabolites is essential to mount an effective resistant state. Earlier accumulation and increased levels greatly potentiates the launching of prompt and effective defence responses *i.e.* in conferring the resistance phenotype, and in the event of the opposite, efficiency of immune responses maybe lessened (Göbel *et al.*, 2001, 2002; Lattanzio *et al.*, 2006; Mazid *et al.*, 2011). Similarly, the accumulation of the apigenin glycosides, more significantly in the NS 5511 cv, possibly

contributed to the cv's state of resistance. The presence of defence-related metabolites (phytoanticipins) in plants creates a state of "readiness" that, in the event of pathogen attack, the metabolites act as a first line of chemical defence to inhibit pathogen proliferation. Additionally, hydrolysis of preformed conjugated phytoanticipins to rapidly generate phytoalexins (Mhlongo *et al.*, 2016), aimed to further limit pathogen proliferation, also contributes to plant resistance (Mazid *et al.*, 2011). Therefore, a rapid increase of antimicrobial metabolite levels significantly contributes to plant resistance phenotype (Lattanzio *et al.*, 2006; Mazid *et al.*, 2011; Balmer *et al.*, 2013; Guptha, 2016). However, noteworthy, the functional roles of phytoanticipins and phytoalexins are somewhat overlapping and can be difficult to clearly distinguish at certain stages of the infection. In a nutshell, the picture depicted by the results from the current study, thus demonstrates metabolic reprogramming (both primary and secondary) in *S. bicolor* following *B. andropogonis* infection and the spanning of an array of defence-related metabolites – aimed at establishing an enhanced defensive capacity (as also shown in the general summary of probable events upon pathogen detection in **Chapter 2**; **Figure 2.6**). Furthermore, the results reveal that the phenylpropanoid and flavonoid metabolic pathways were central in *S. bicolor* defence against to *B. andropogonis*.

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Chapter 4: Supplementary Material

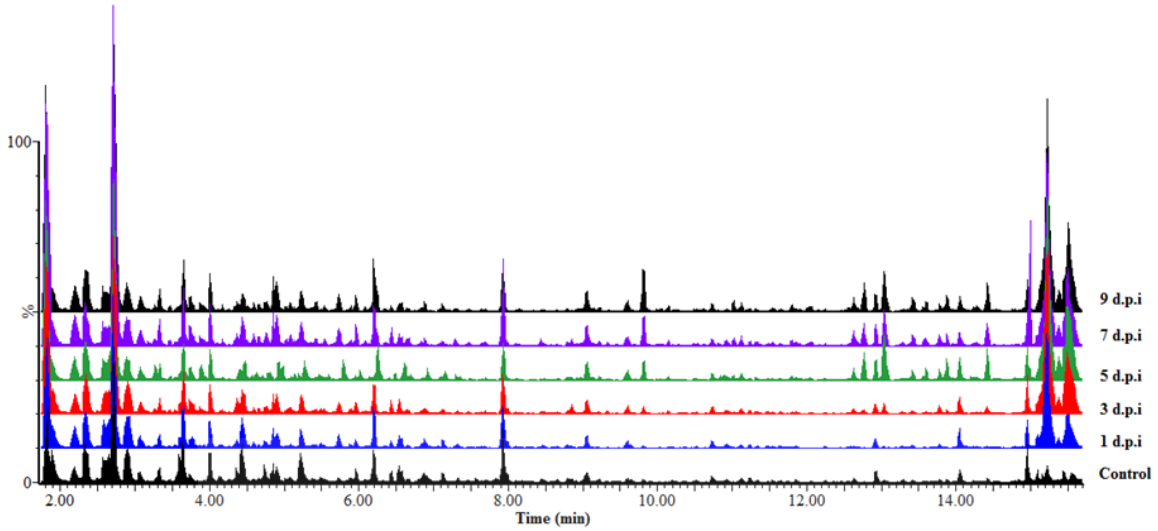


Figure S 4.1: UHPLC-MS BPI chromatograms for ESI positive data of sorghum NS 5511 (BT) cv samples. A control (non-treated) sample extract chromatogram is shown against treated samples (1 - 9 d.p.i.) extracts chromatograms. It can be seen from chromatograms that the sample extracts contained a mixture of polar, mid-polar and non-polar compounds, with more of the compounds being mid-polar. The chromatograms also show visually noticeable differences between the control sample and treated samples as well as time-dependent metabolic changes.

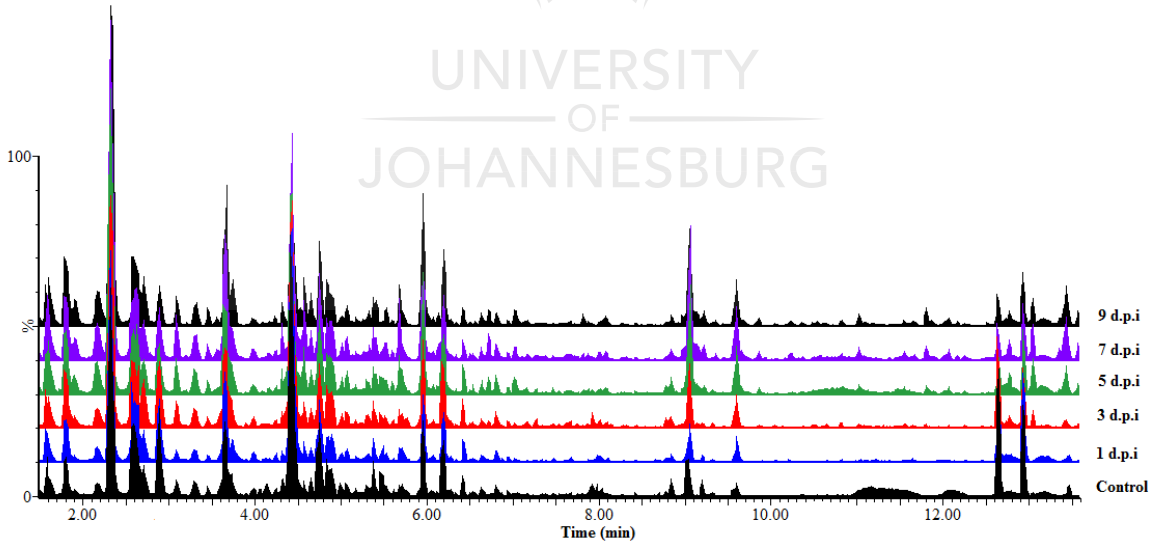


Figure S 4.2: UHPLC-MS BPI chromatograms for ESI negative data of sorghum NS 5655 (ST) cv samples. A control (non-treated) sample extract chromatogram is shown against treated samples (1 - 9 d.p.i.) extracts chromatograms. It can be seen from chromatograms that the sample extracts contained a mixture of polar, mid-polar and non-polar compounds, with more of the compounds being mid-polar. The chromatograms also show visually noticeable differences between the control sample and treated samples as well as time-dependent metabolic changes.

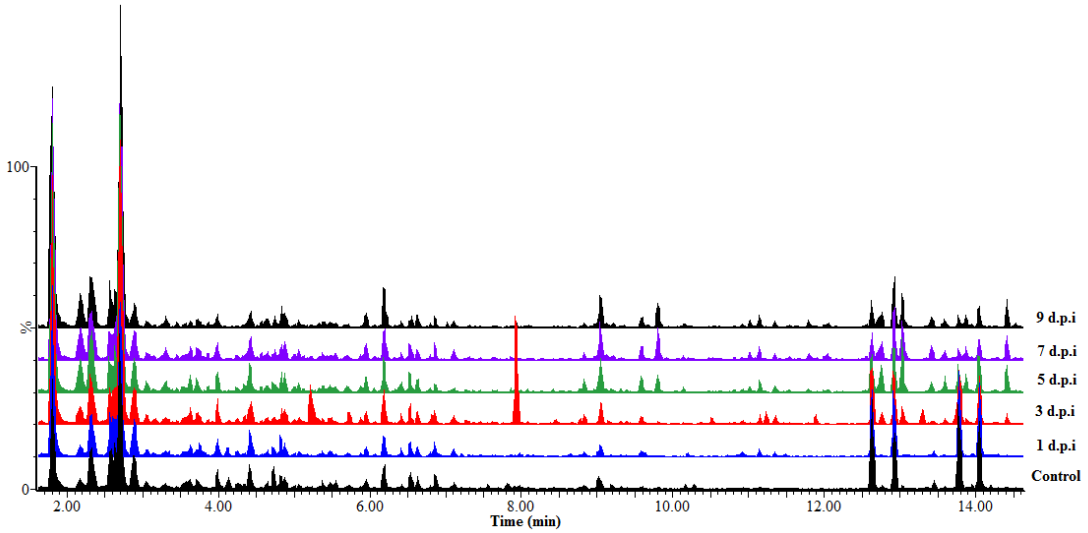


Figure S 4.3: UHPLC-MS BPI chromatograms for ESI positive data of sorghum NS 5655 (ST) cv samples. A control (non-treated) sample extract chromatogram is shown against treated samples (1 - 9 d.p.i.) extracts chromatograms. It can be seen from chromatograms that the sample extracts contained a mixture of polar, mid-polar and non-polar compounds, with more of the compounds being mid-polar. The chromatograms also show visually noticeable differences between the control sample and treated samples as well as time-dependent metabolic changes.

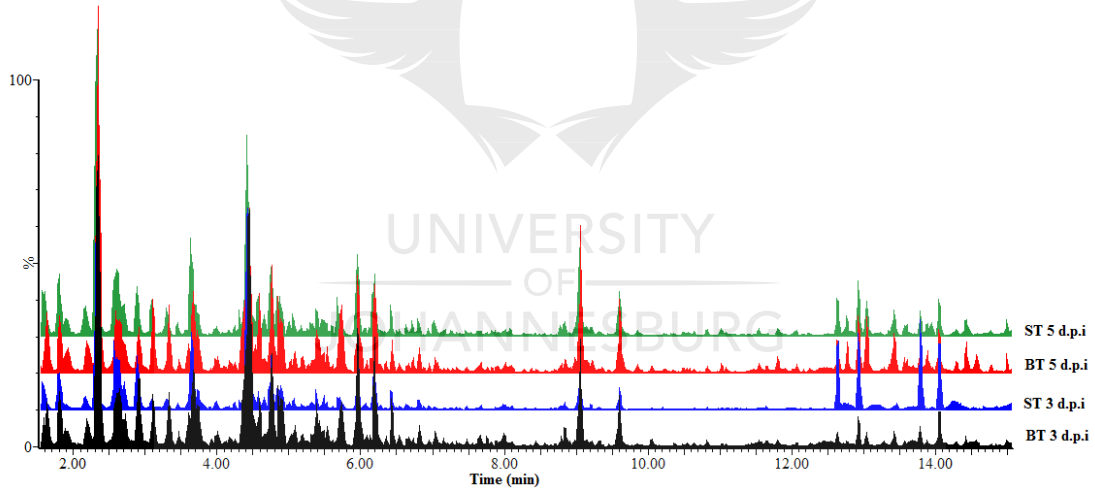


Figure S 4.4: UHPLC-MS BPI chromatograms for ESI negative data of sorghum NS 5511 (BT) vs NS 5655 (ST) cv samples. BT treated samples chromatograms 3 and 5 d.p.i. are shown against ST treated samples 3 and 5 d.p.i.. The chromatograms visually show cultivar-dependent differences as well as time-dependent metabolic changes.

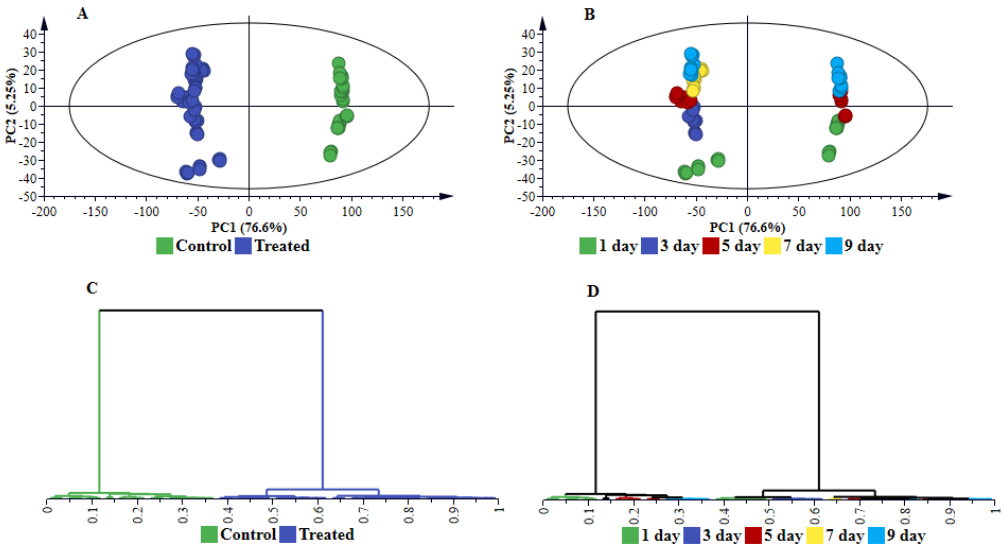


Figure S 4.5: PC and HC analyses of the ESI positive data for sorghum NS 5511 (BT) extracts (excluding the QC data). **(A & B)** A 10-component model, explaining 92.1% variations in Pareto-scaled data, X, and the amount of predicted variation by the model, according to cross-validation, is 86.7%. **A** and **B** is the same scores plot, with **A** coloured according to treatment and **B** coloured according to time. This two-dimensional scores space, spanned by the first two PCs, reveals treatment-related sample clustering (treated = blue, controls/non-treated = green) and also time-related clustering. **(C & D)** HCA dendrograms computed from low dimensional data (**PC scores plot**). The models were computed using 10 vectors/components, explaining 92.1% of the total variation in X matrix. **C** displays treatment related relationships amongst the samples, treated (blue) samples from both cultivars are clearly separated from the non-treated samples (green) except for a few samples. While **D** displays time related relationships.

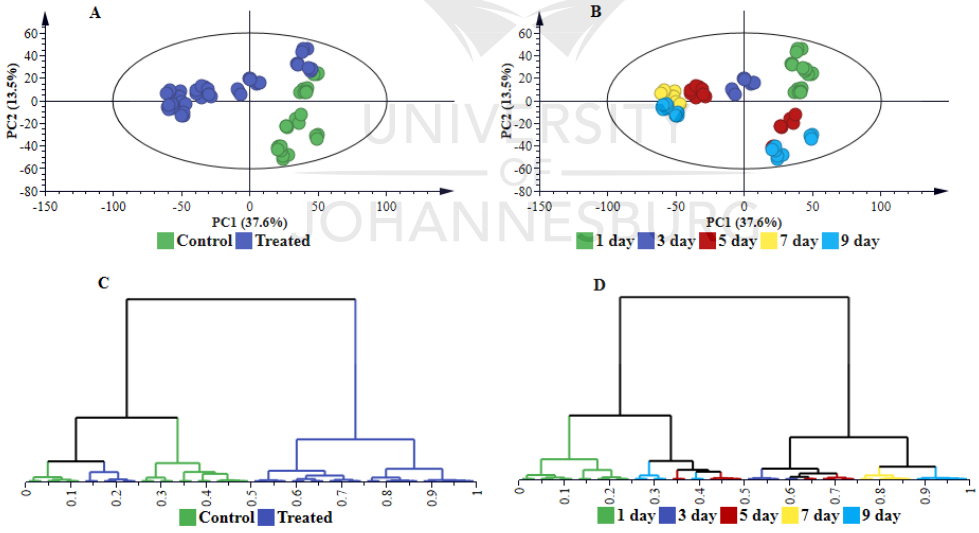


Figure S 4.6: PC and HC analyses of the ESI negative data for sorghum NS 5655 (ST) extracts (excluding the QC data). **(A & B)** A 8-component model, explaining 71.0% variations in Pareto-scaled data, X, and the amount of predicted variation by the model, according to cross-validation, is 60.4%. **A** and **B** is the same scores plot, with **A** coloured according to treatment and **B** coloured according to time. This two-dimensional scores space, spanned by the first two PCs, reveals treatment-related sample clustering (treated = blue, controls/non-treated = green) and also time-related clustering. **(C & D)** HCA dendrograms computed from low dimensional data (**PC scores plot**). The models were computed using 8 vectors/components, explaining 71.0% of the total variation in X matrix. **C** displays treatment related relationships amongst the samples, treated (blue) samples from both cultivars are clearly separated from the non-treated samples (green) except for a few samples. While **D** displays time related relationships.

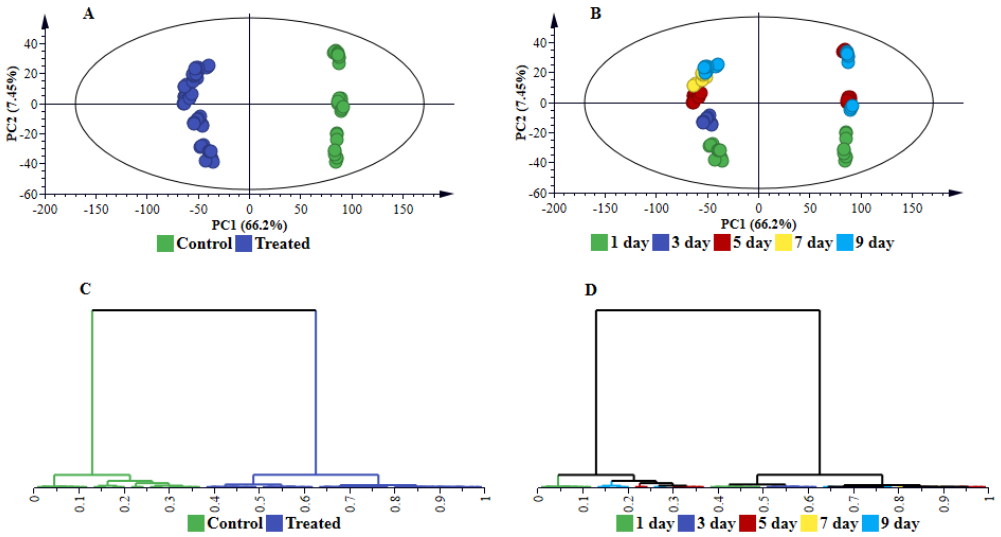


Figure S 4.7: PC and HC analyses of the ESI positive data for sorghum NS 5655 (ST) extracts (excluding the QC data). **(A & B)** A 5-component model, explaining 82.0% variations in Pareto-scaled data, X, and the amount of predicted variation by the model, according to cross-validation, is 78.0%. **A** and **B** is the same scores plot, with **A** coloured according to treatment and **B** coloured according to time. This two-dimensional scores space, spanned by the first two PCs, reveals treatment-related sample clustering (treated = blue, controls/non-treated = green) and also time-related clustering. **(C & D)** HCA dendrograms computed from low dimensional data (**PC scores plot**). The models were computed using 7 vectors/components, explaining 82.0% of the total variation in X matrix. **C** displays treatment related relationships amongst the samples, treated (blue) samples from both cultivars are clearly separated from the non-treated samples (green) except for a few samples. While **D** displays time related relationships.

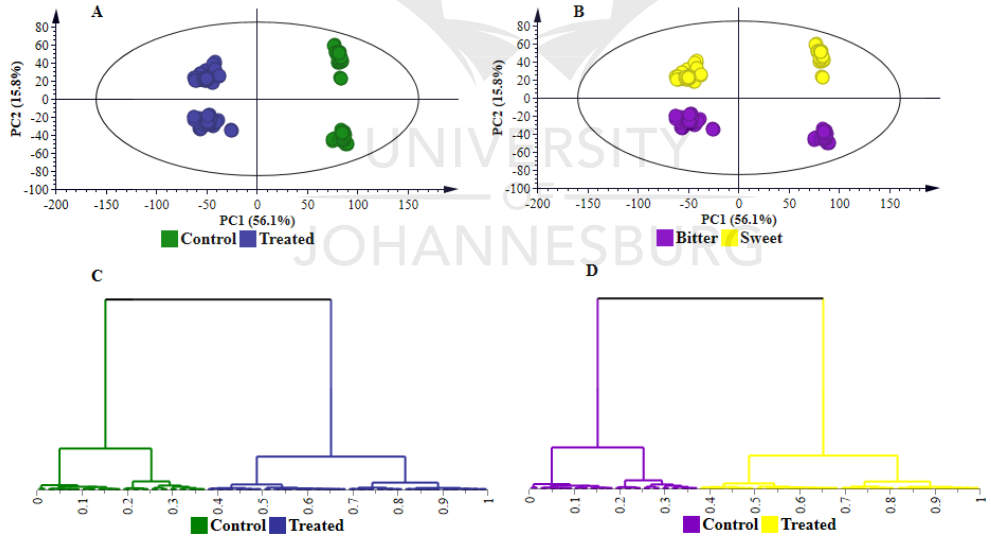


Figure S 4.8: PC and HC analyses of the ESI positive data for sorghum NS 5511 (BT) and NS 5655 (ST) extracts (excluding the QC data). **(A & B)** A 14-component model, explaining 91.8% variation in Pareto-scaled data, X, and the amount of predicted variation by the model, according to cross-validation, is 87.7%. . **A** and **B** is the same scores plot, with **A** coloured according to treatment and **B** coloured according to cultivar. This two-dimensional scores space, spanned by the first two PCs, reveals treatment-related sample clustering (treated = blue, controls/non-treated = green) and also cultivar-related clustering. **(C & D)** HCA dendrograms computed from low dimensional data (**PC scores plot**). The models were computed using 14 vectors/components, explaining 91.8% of the total variation in X matrix. **C** displays treatment related relationships amongst the samples, treated (blue) samples from both cultivars are clearly separated from the non-treated samples (green) except for a few samples. While **D** displays cultivar related relationships.

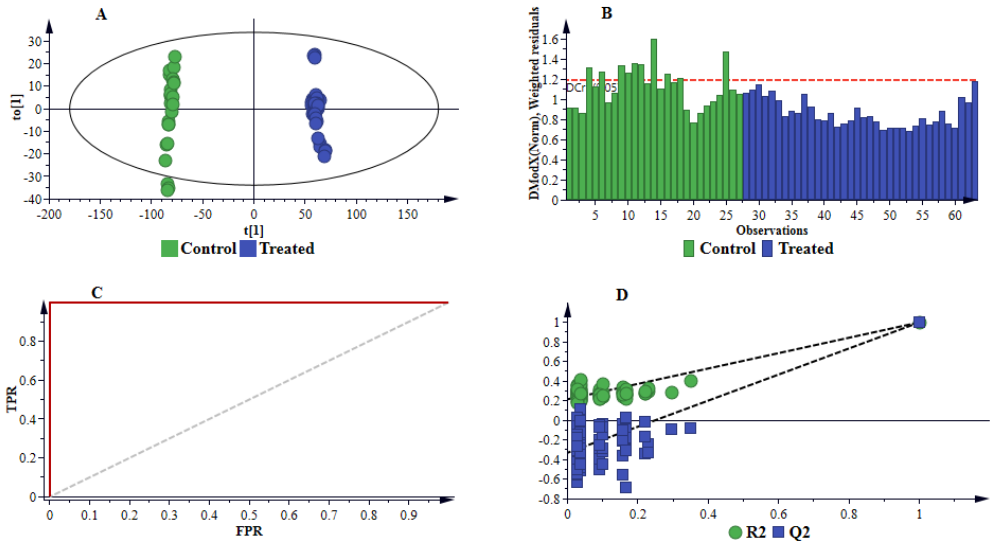


Figure S 4.9: Supervised multivariate analyses of the ESI positive data for NS 5511 (BT) cv extracts (excluding QCs). (A) The OPLS-DA score plot shows grouping of control vs treated for all samples. This model comprises 1 predictive component and 1 orthogonal components (R^2X= 81.2%, R^2Y= 99.8% and Q^2= 98.8%). **(B)** A distance to the model in space X (DModX) plot showing moderate outliers (above the dashed red line, Dcrit) in the OPLS-DA scores plot. (**C**) A representative receiver operator characteristic (ROC) plot summarising the performance of OPLS-DA (a binary classification method). **(D)** The response permutation test plot (n=100) of the OPLS-DA model in A, clearly separating control from treated plant samples.

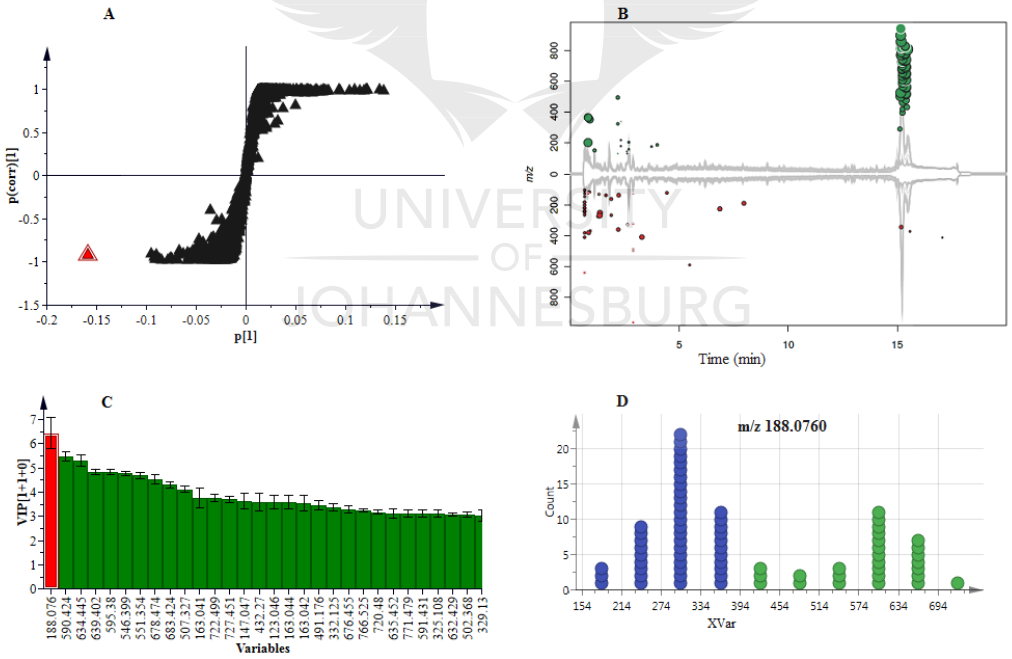


Figure S 4.10: OPLS-DA modelling analyses of the ESI positive data for NS 5511 (BT) cv extracts (excluding QCs). (A) OPLS-DA loading S-plot displays the discriminating features (ions) that explain the clustering (sample grouping) observed in the OPLS-DA scores plot with the features in the top right quadrant are positively correlated to the treatment and those in the bottom left quadrant are negatively correlated to the treatment. This loading S-plot comprises 1 predictive component explaining 78.4% of the total variation and 1 orthogonal components explaining 2.79% of the total variation. **(B)** XCMS cloud plot. (**C**) A VIP plot summarising the importance of variables in the projection of the PLS model. A VIP value >1 is significant/important in the projection and increase in value indicates an increase in significance of the variable. **(D)** Dot plot of the selected variable **188.0760** m/z from S-plot (in red; also marked on the VIP plot) showing no overlap between control and treated groups thus, the variable strongly discriminates the two groups.

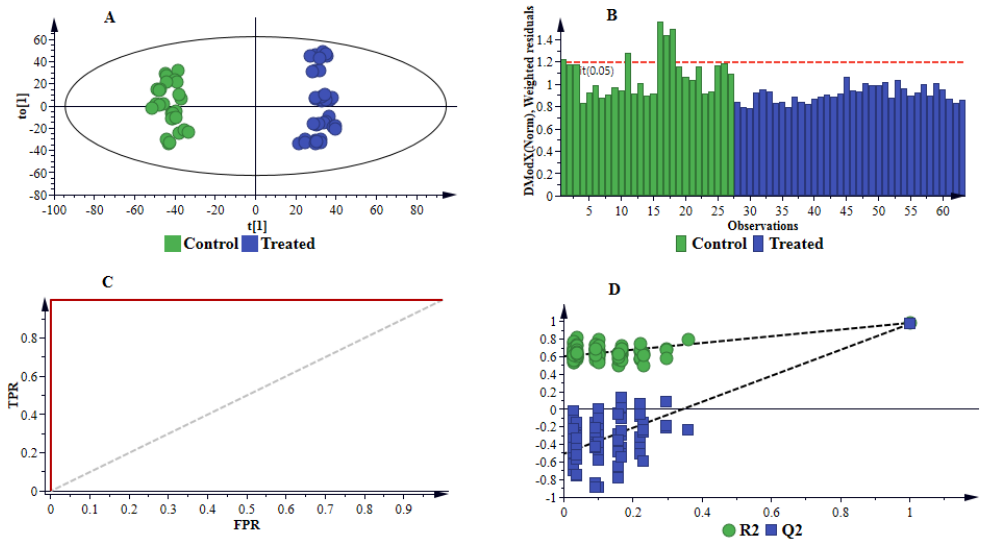


Figure S 4.11: Supervised multivariate analyses of the ESI negative data for NS 5655 (ST) cv extracts (excluding QCs). (A) The OPLS-DA score plot shows grouping of control vs treated for all samples. This model comprises 1 predictive component and 2 orthogonal components (R^2X= 56.0%, R^2Y= 98.7% and Q^2= 97.6%). **(B)** A distance to the model in space X (DModX) plot showing moderate outliers (above the dashed red line, Dcrit) in the OPLS-DA scores plot. (**C**) A representative receiver operator characteristic (ROC) plot summarising the performance of OPLS-DA (a binary classification method). **(D)** The response permutation test plot (n=100) of the OPLS-DA model in A, clearly separating control from treated plant samples.

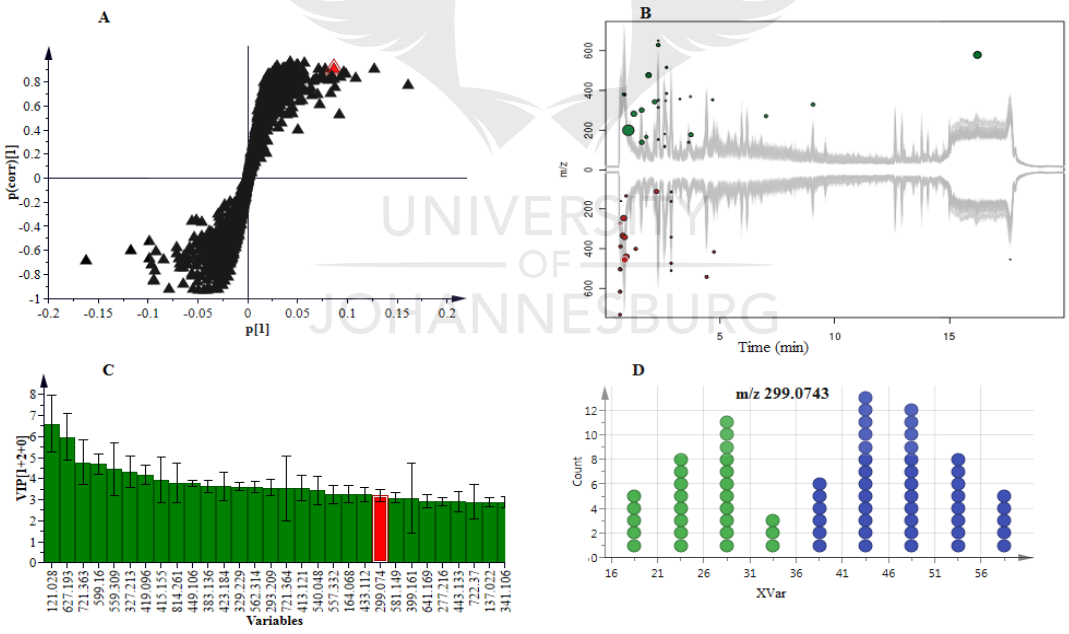


Figure S 4.12: OPLS-DA modelling analyses of the ESI negative data for NS 5655 (ST) cv extracts (excluding QCs). (A) OPLS-DA loading S-plot displays the discriminating features (ions) that explain the clustering (sample grouping) observed in the OPLS-DA scores plot with the features in the top right quadrant are positively correlated to the treatment and those in the bottom left quadrant are negatively correlated to the treatment. This loading S-plot comprises 1 predictive component explaining 34.4% of the total variation and 2 orthogonal components explaining 22.0% of the total variation. **(B)** XCMS cloud plot. (**C**) A VIP plot summarising the importance of variables in the projection of the PLS model. A VIP value >1 is significant/important in the projection and increase in value 1 indicates an increase in significance of the variable. **(D)** Dot plot of the selected variable **299.0743** m/z from S-plot (in red; also marked on the VIP plot) showing no overlap between control and treated groups thus, the variable strongly discriminates the two groups.

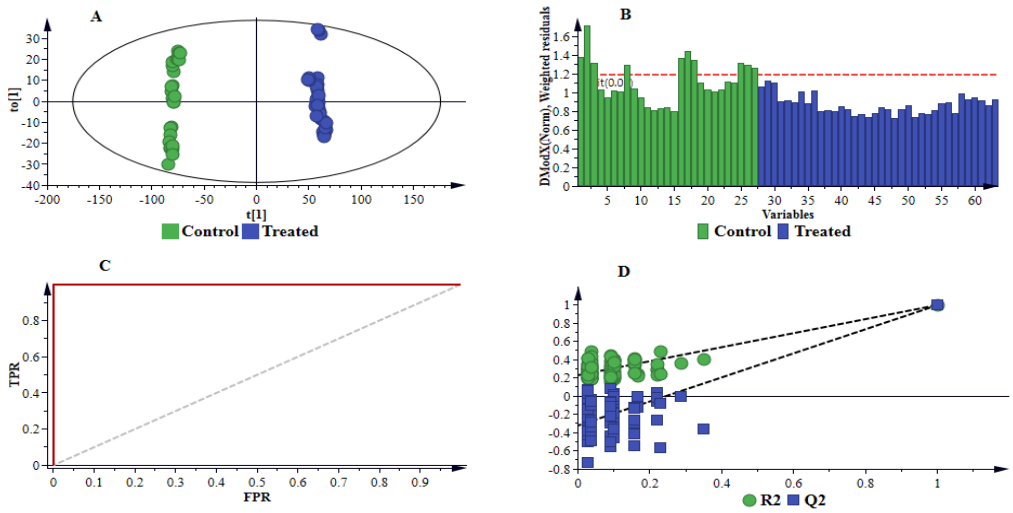


Figure S 4.13: Supervised multivariate analyses of the ESI positive data for NS 5655 (ST) cv extracts (excluding QCs). (A) The OPLS-DA score plot shows grouping of control vs treated for all samples. This model comprises 1 predictive component and 1 orthogonal components (R^2X= 71.7.0%, R^2Y= 99.7% and Q^2= 99.6%). **(B)** A distance to the model in space X (DModX) plot showing moderate outliers (above the dashed red line, Dcrit) in the OPLS-DA scores plot. (**C**) A representative receiver operator characteristic (ROC) plot summarising the performance of OPLS-DA (a binary classification method). **(D)** The response permutation test plot (n=100) of the OPLS-DA model in A, clearly separating control from treated plant samples

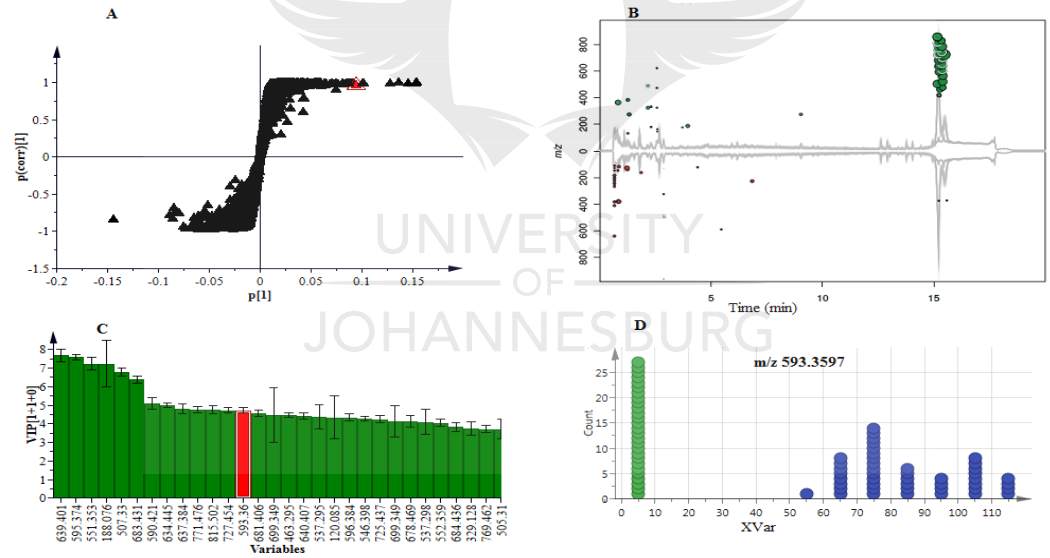


Figure S 4.14: OPLS-DA modelling analyses of the ESI positive data for NS 5655 (ST) cv extracts (excluding QCs). (A) OPLS-DA loading S-plot displays the discriminating features (ions) that explain the clustering (sample grouping) observed in the OPLS-DA scores plot with the features in the top right quadrant are positively correlated to the treatment and those in the bottom left quadrant are negatively correlated to the treatment. This loading S-plot comprises 1 predictive component explaining 68.4% of the total variation and 1 orthogonal components explaining 3.32% of the total variation. **(B)** XCMS cloud plot. (**C**) A VIP plot summarising the importance of variables in the projection of the PLS model. A VIP value >1 is significant/important in the projection and increase in value indicates an increase in significance of the variable. **(D)** Dot plot of the selected variable **593.3597** m/z from S-plot (in red; also marked on the VIP plot) showing no overlap between control and treated groups thus, the variable strongly discriminates the two groups.

Chapter 5: Results and Discussion, part II: The effect of purified LPS from *Burkholderia andropogonis* on suspension-cultured *Sorghum bicolor* cells

As detailed in the literature review (**Chapter 2**; **section 2.3**), passive (preformed) and active (induced) immune responses in plants exposed to pathogens are triggered upon detection of conserved molecular motifs such as LPS, flagellin and fungal chitin amongst other well-characterised MAMPS (refer to cited literature for detailed description) (Chinchilla *et al.*, 2007; Wan *et al.*, 2008; Hao *et al.*, 2014; Shamrai, 2014; Ranf *et al.*, 2015; Ranf, 2016). In the previous chapter (**Chapter 4**) the metabolic response of sorghum plants to *B. andropogonis* infection were highlighted. However, there is a need to further investigate the important factors instrumental to contributing to the resulting metabolic reprogramming. In this attempt, the effect of purified LPS isolated from *B. andropogonis*, as one of the pathogen's MAMPs triggering the metabolic reprogramming and the extent to which this occurs, was investigated using a cell suspension culture system. The latter was used to gain more detailed insights into the molecular mechanisms employed by sorghum in response to infection. Additionally, this system was employed, due to rapid multiplication of a homogeneous population of cells, decreased biological variability and improved experimental reproducibility, advantageous for the study of secondary metabolite biosynthetic pathways and inducible defence responses (also see **Chapter 3**; **section 3.2**) (Coventry, 1999; Bourgaud *et al.*, 2001; Ngara *et al.*, 2008; Ellis & Goodacre, 2012; Tugizimana, 2012).

5.1. Compositional - and structural analysis of LPS from *Burkholderia andropogonis*

LPS from the *B. andropogonis* ($LPS_{B.\ andr.}$) is a macromolecular (M_r > 7.5 kDa), hydrophilic hetero-polysaccharide, that is covalently linked through a core oligosaccharide (M_r

approximately 1.8 kDa) to the glycolipid moiety, Lipid A (M_r approximately 1.6 kDa). An LPS-specific SDS-PAGE analysis of purified $LPS_{B.\ andr.}$ is shown in **Figure 5.1**. The lanes on the gel loaded with purified LPS display a characteristic ladder pattern with the individual bands representing $LPS_{B.\ andr.}$ of various molecular sizes, *i.e.* size heterogeneity. This can be ascribed to biosynthesis products differing in length, *i.e.*, consisting of the Lipid A and core oligosaccharide, and containing a different number of oligosaccharide repeating units that forms the extended O-polysaccharide (OPS).

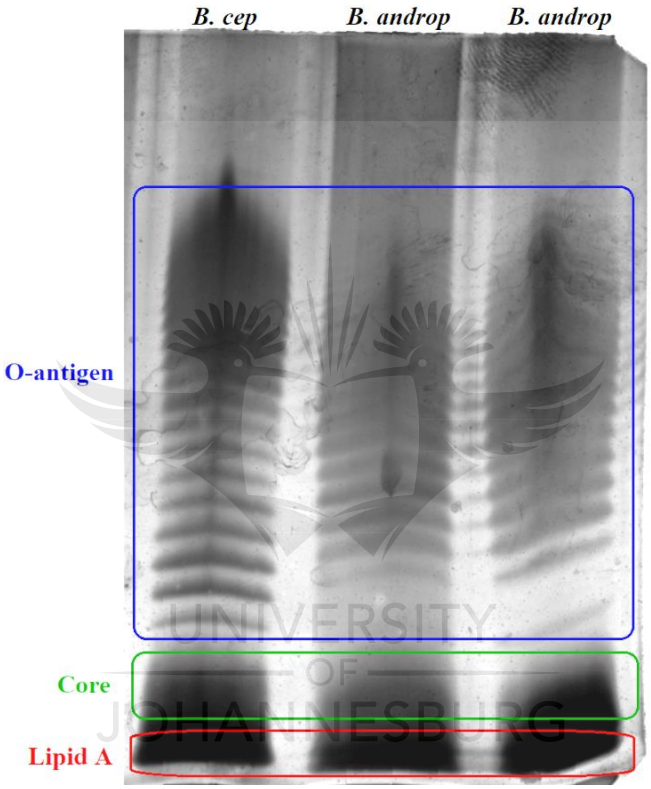


Figure 5.1: LPS-specific SDS-PAGE analysis of purified $LPS_{B.\ andr.}$. LPS isolated from *Burkholderia andropogonis* (last two lanes) is displayed against *Burkholderia cepacia* LPS (first lane). $LPS_{B.cep.}$ was used as a reference as it has been well-characterised (Madala *et al.*, 2011). The highlighted regions show the three LPS components from the two *Burkholderia* species. The Lipid A component of $LPS_{B.\ andr.}$ appears at the bottom of the gel (~ 1.6 kDa; region highlighted in red) and is attached to the core oligosaccharide – mid section (~ 1.8 kDa; region highlighted in green). The region highlighted in blue shows the repeating units of the O-antigen/O-polysaccharide (OPS; heterogeneous in size).

Characterisation of the purified LPS was performed in the Department of Chemical Sciences, University of Napoli Federico II, Naples, Italy (Di Lorenzo and Molinaro, unpublished). Sugar compositional analysis revealed the following: rhamnose, 3-*C*-methylrhamnose, 2,6-dideoxy-2-amino-D-glucose (quinovosamine), glucosamine, 4-amino-4-deoxy-L-arabinose (Ara4N), D-glucose, D-galactose, L-*glycero*-D-*manno*-heptose, 3-deoxy-D-*manno*-oct-2-

ulopyranosonic acid (Kdo), and D-*glycero*-D-*talo*-oct-2-ulopyranosonic acid (Ko). The repeating unit of the OPS of the $LPS_{B.\ andr.}$ was characterised as having a [→3)-α-Rha-(1→2)-α-Rha3*C*Me-(1→3)-α-Rha-(1→] motif.

Structural analysis on the Lipid A component of $LPS_{B.\ andr.}$ revealed a penta-acylated, 1,4'-*bis*-phosphorylated, [β-D-GlcpN-(1→6)-α-D-GlcpN] disaccharide backbone, further substituted by 4-amino-4-deoxy-L-arabinopyranose (L-Ara4N) at the anomeric position of GlcN by a phosphodiester linkage. Fatty acid analysis indicated the presence of (*R*)-3-hydroxyhexadecanoic acid (16:0(3-OH)), (*R*)-3-hydroxytetradecanoic acid (14:0(3-OH)) and tetradecanoic acid (14:0). As primary fatty acids, R-configurated 16:0(3-OH) (amide-linked in 2 and 2′) and 14:0(3-OH) (ester-linked in 3 and 3′) were identified. A secondary 14:0 was located at position 2′. Both fatty acids and Ara4N were not in stoichiometric amounts; indeed, the $LPS_{B.\ andr.}$ Lipid A was naturally present as a 'blend' of different species differing by the presence or absence of fatty acids and further complicated by the non-stoichiometric presence of Ara4N (**Figure 5.2**) (Di Lorenzo and Molinaro, unpublished).

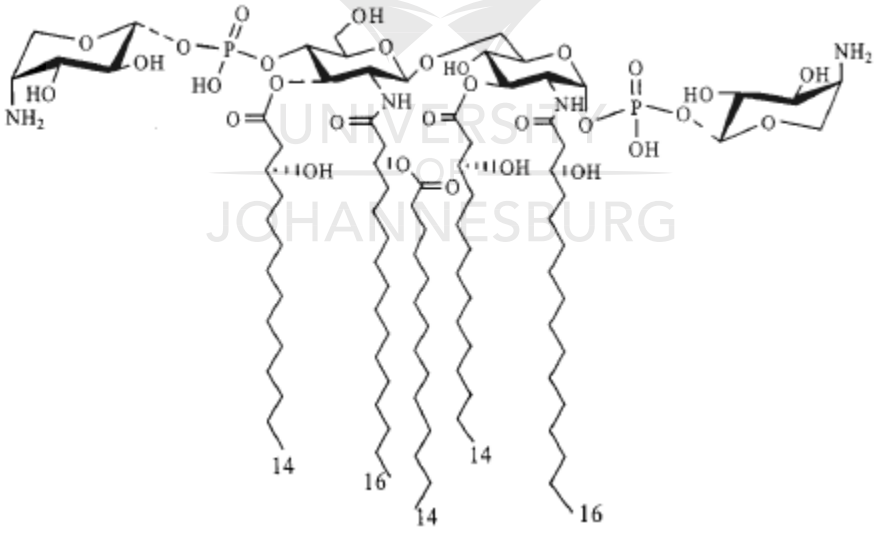


Figure 5.2: Structural representation of the Lipid A component of *B. andropogonis* LPS. The Mr of the Lipid A component is approximately 1.6 kDa. Structural analysis revealed that the Lipid A is composed of a penta-acylated, 1,4'-*bis*-phosphorylated disaccharide backbone, which is further substituted by 4-amino-4-deoxy-L-arabinopyranose at the anomeric position of GlcN by a phosphodiester linkage and fatty acid analysis revealed the presence of (*R*)-3-hydroxyhexadecanoic acid, (*R*)-3-hydroxytetradecanoic acid and tetradecanoic acid. Lipid A is regarded a highly conserved component, differing amongst bacterial species and contributing to LPS stability.

5.2. A non-targeted metabolic profiling of LPS*B. andr.*-treated cultured sorghum cells

High definition LC-MS-based metabolomics approaches have paved ways into the uncovering of metabolite profiles of various biosystems, to a greater depth (Tugizimana *et al.*, 2013; Ncube *et al.*, 2017). LC-MS analysis employing ESI ionisation, has allowed a more comprehensive analysis of plant metabolites differing in physicochemical properties (Xiao *et al.*, 2012; Putri *et al.*, 2013; Yin & Xu, 2014; Wang *et al.*, 2015; Tugizimana *et al.*, 2017). In this study UHPLC-ESI-QTOF-HDMS was employed for the analysis of endo- and exometabolome of sorghum cell suspension cultures. BPI chromatograms of these extracts displayed treatment- and time-related metabolic responses to LPS*B. andr.* treatment. Differences with regard to peak population and peak intensities across the BPI chromatograms of treated and non-treated cell extracts were chromatographically visualised. A clear example of variation in peak population and intensities across samples can be seen in the marked regions *i.e.* Rt = 12.4-13.6 min and Rt = 8.5-9.5 min of MS chromatograms obtained from extracts of cell (**Figure 5.3**) and growth medium (**Figure 5.4**), showing the comparison of non-treated (control, 0 h) and treated samples (12-30 h). The differences between control *vs* treated samples and those across the time points, subsequently provided the visual picture of metabolic changes occurring due to the LPS*B. andr.* treatment as a function of time. However, these differences were not as pronounced as those visually noticed in comparable MS chromatograms of extracts prepared from leaf tissue (**Chapter 4**, **section 4.2**).

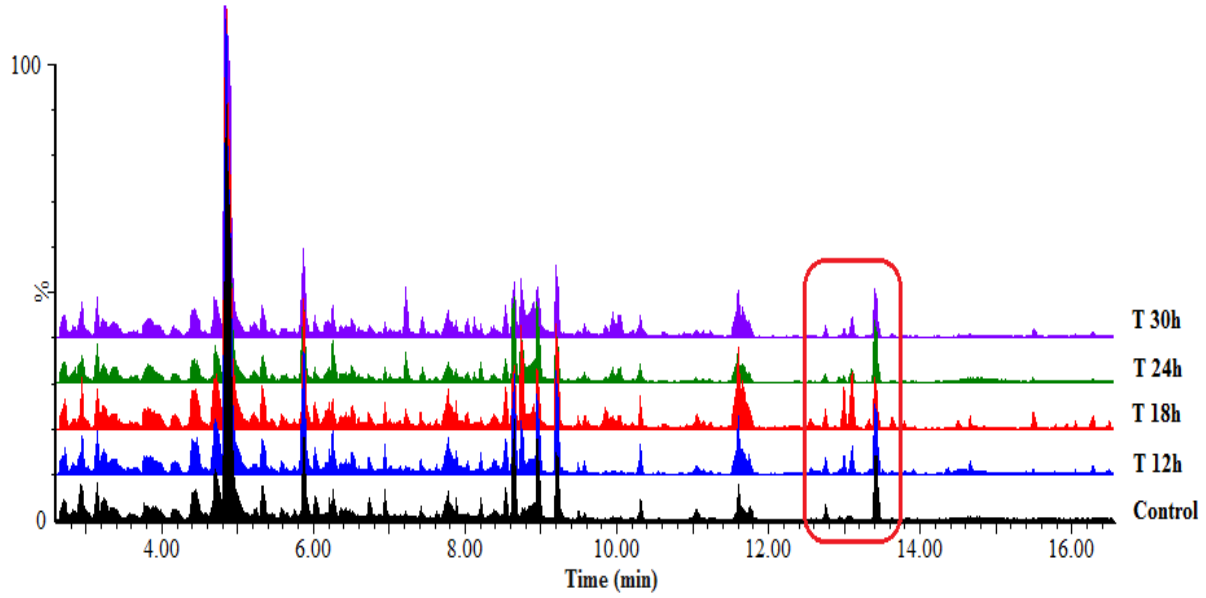


Figure 5.3: UHPLC-MS BPI chromatograms (ESI negative) of methanolic <u>intracellular</u> extracts of sorghum cells treated with LPS*B. andr.* The chromatograms of a control (non-treated 0 h) *vs* treated samples (12-30 h) display variation related to treatment- and time-related metabolic changes occurring in the cells due to LPS treatment.

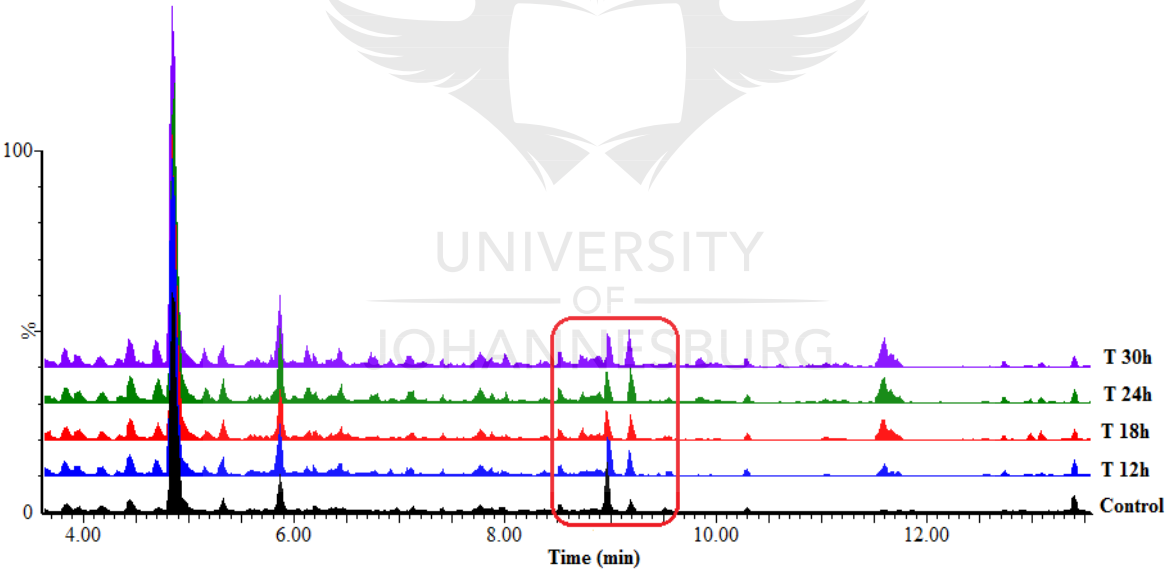


Figure 5.4: UHPLC-MS BPI chromatograms (ESI negative) of methanolic <u>extracellular</u> extracts of sorghum cells treated with LPS*B. andr.* The chromatograms of a control (non-treated 0 h) *vs* treated samples (0-30 h) display variation related to treatment- and time-related metabolic changes occurring in the cells due to LPS treatment.

BPI chromatograms only indicate the most intense peaks at a specific Rt and are not clearly informative in providing a holistic view of the variation in samples. Multivariate statistical analyses (unsupervised and supervised) were therefore performed to further investigate the treatment- and time-related differences observed from the LC-MS analyses. In unsupervised

multivariate analyses, PCA as one of the chemometrics tools, provided a summary of the multidimensional data, increased data interpretability and permitted recognition of groupings, trends and outliers – while virtually preserving statistical variability (Ivosev G. *et al.*, 2008; Tugizimana *et al.*, 2013; Jolliffe *et al.*, 2016; Schwarz *et al.*, 2018). The computed PCA scores plot of cell extracts revealed a clear separation between control and treated samples (**Figure 5.5 A** and **S 5.3 A**). A similar clustering was also observed for the medium extracts **(Figure 5.6 A** and **S 5.4 A**). These groupings revealed treatment-related sample clustering, reflecting differences highlighted in the BPI chromatograms (**Figure 5.3-5.4** and **S 5.1-5.2**).

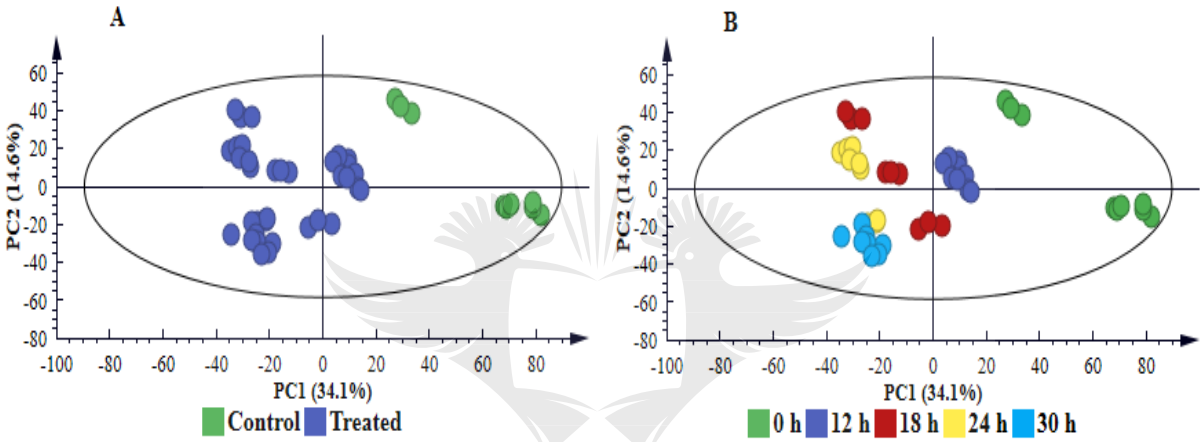


Figure 5.5: PC analyses of the LC-MS (ESI negative data) for <u>intracellular</u> sorghum cell extracts. The 4-component model, explains 61.9% variation in Pareto-scaled data, X, and the amount of predicted variation by the model, according to cross-validation, is 51.4%. The first 2 PCs were used to generate the above scores plot. **A:** Clusters coloured based on condition *i.e.* non-treated/treated shows clear separation between treated and control (non-treated, 0 h) samples. **B** is the same scores plot but coloured according to time shows a clear sequential time trend clustering (from C0 h and T12-30 h). To determine the group (control/treated) to which the clusters in **B** belong, link to the corresponding positions in **A**.

When sample groups were colour-coded base on time points, the same scores plots showed a distinct sequential time clustering trend of samples *i.e.* a time-related clustering for both cell (**Figure 5.5 B** and **S 5.3 B**) and medium extracts (**Figure 5.6 B** and **S 5.4 B**). Clustering of samples highlighted in the PCA scores space hints to differential metabolic changes in cultured sorghum cells in response to $LPS_{B.\ andr.}$ treatment.

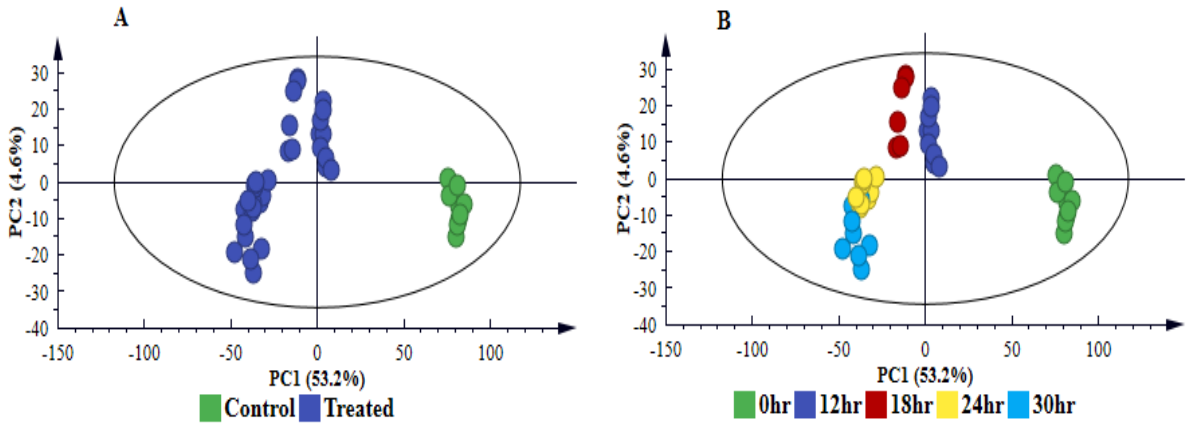


Figure 5.6: PC analyses of the LC-MS (ESI negative data) for extracellular sorghum cell extracts. The 3-component model, explains 61.5% variation in Pareto-scaled data, X, and the amount of predicted variation by the model, according to cross-validation, is 52.8%. First 2 PCs were used to generate the above scores scatter plot. **A:** Clusters coloured based on condition *i.e.* non-treated/treated shows clear separation between treated and control (non-treated). **B: S**ame scores plot, but coloured according to time points. A clear sequential time trend clustering (from C0 h and T12-30 h) can be seen in **B**. To determine the group (control/treated) to which the clusters in **B** belong, link to the corresponding positions in **A**.

For further characterisation and interpretation of the different clustering depicted in PC analyses, a supervised method, OPLS-DA, was applied. The supervised modelling allowed identification of signatory biomarkers underlying the discrimination between the sample classes (associated with LPS treatment). Computed OPLS-DA scores plots showed clear discrimination between the control and the treated samples (**Figure 5.7-5.8 A** and **Figure S 5.5-5.6 A**). Evaluation of the goodness-of-fit ($R^2X(cum)$), proportion of variance of the response variable explained by the model ($R^2Y(cum)$) and predictive ability ($Q^2(cum)$) parameters – indicated that the models were reliable. Further validation also revealed the reliability of the models *i.e.* CV-ANOVA p-value of ≤ 0.05. (Fujimura *et al.*, 2011; Li *et al.*, 2014; Tugizimana *et al.*, 2014, 2015; Ncube *et al.*, 2016). Furthermore, the distance to the model in space X (DModX) plots (**Figure 5.7-5.8 B** and **S 5.5-5.6 B**) for the generated OPLS-DA scores plots were used to assess moderate outliers.

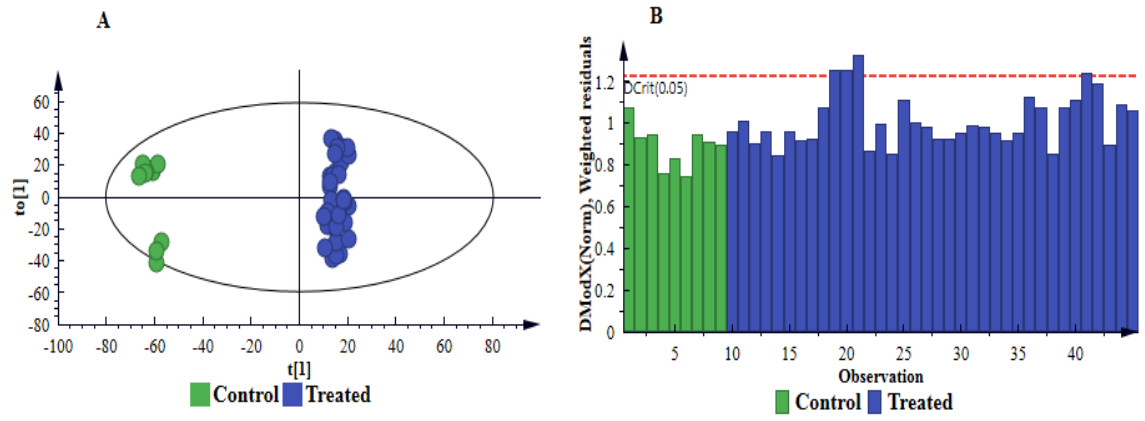


Figure 5.7: Supervised multivariate analyses of the LC-MS (ESI negative data) for <u>intracellular</u> extracts. (A): Grouping of control (C0 h) *vs* treated (all time points combined) as indicated by an OPLS-DA score plot. This model comprises 1 predictive component and 3 orthogonal components (R^2X= 60.0%, R^2Y= 99.2% and Q^2= 95.1%). **(B):** A distance to the model in space X (DModX) plot to detect outliers (above the dashed red line, Dcrit).

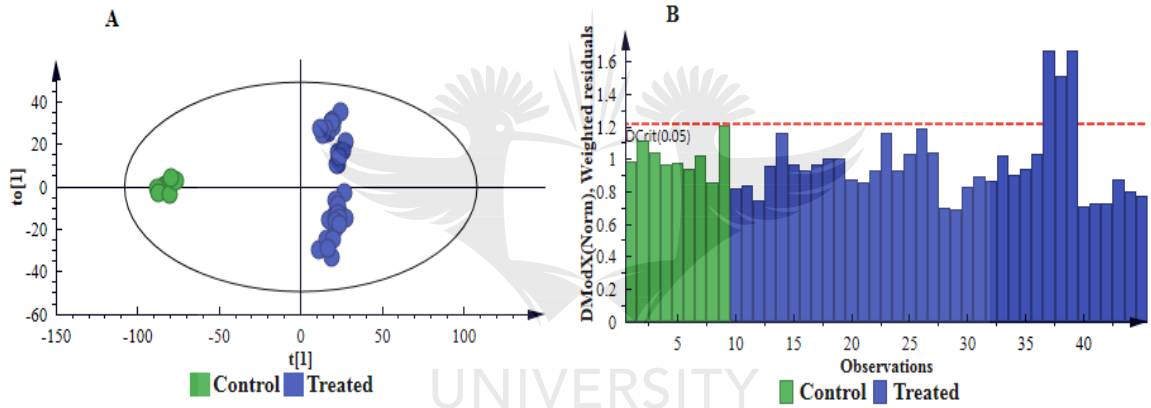


Figure 5.8: Supervised multivariate analyses of the LC-MS (ESI negative data) for <u>extracellular</u> extracts (excluding QCs). (A): Grouping of control (C0 h) *vs* treated (all time points combined) as indicated by an OPLS-DA score plot. This model comprises 1 predictive component and 1 orthogonal components (R^2X= 53.8%, R^2Y= 99.1% and Q^2= 96.6%). **(B):** A distance to the model in space X (DModX) to detect outliers (above the dashed red line, Dcrit).

The OPLS-DA derived loadings S-plot (**Figures 5.9-5.10 A** and **S 5.7-5.8 A**) assisted in visualisation of the covariance and correlation between variables of modelled classes, and permitted the extraction of statistically significant biomarkers within the $|p[1]| \geq 0.05$ and $|p(corr)| \geq 0.5$ defined regions, responsible for discrimination between control and $LPS_{B.\ andr.}$ treated samples (Major *et al.*, 2006; Fujimura *et al.*, 2011; Tugizimana *et al.*, 2014, 2017). The significance of the extracted variables towards discrimination of samples was assessed using the VIP plots. Only variables with a VIP score > 1 were considered significant and thus, further annotated (Pears *et al.*, 2005; Fujimura *et al.*, 2011; Li *et al.*, 2014). **Figures 5.9-5.10 B** (and **Figures S 5.7-5.8 B**) display some of the variables considered as significant, with the one

highlighted in red corresponding to the selected variable (in red) in the S-plot (**Figures 5.9-5.10 A**).

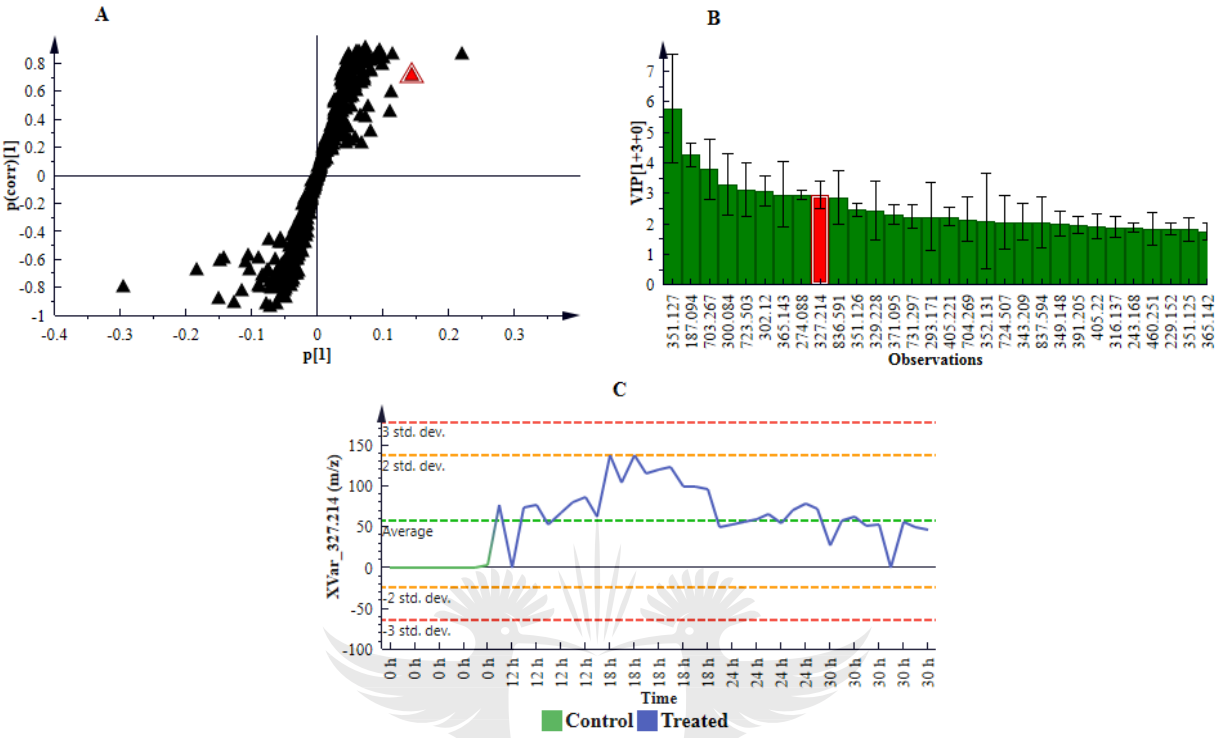


Figure 5.9: OPLS-DA modelling analyses of the LC-MS (ESI negative data) for <u>intracellular</u> extracts (excluding QCs). (A) An OPLS-DA loading S-plot displaying the discriminating features (ions) that explain the clustering (sample grouping) observed in the OPLS-DA scores plot with the features in the top right quadrant positively correlated to the treatment and those in the bottom left quadrant negatively correlated to the treatment. **(B)** A VIP plot summarising the importance of some of the variables in the projection of the model with the *m/z* values and jackknife confidence intervals reflecting the variable stability. A VIP value >1 indicates a significant variable in the complex analysis in comparing the difference between groups. **(C)** Representative variable trend plot exhibiting the trend of the selected variable across the control and treated samples.

Moreover, an additional statistical tool, variable trend plot was also used for variable selection evaluation. Representative variable trend plots (**Figures 5.9-5.10 C** and **S 5.7-5.8 C)** for the variable highlighted (in red) in the S-plots and VIP plot, exhibit changes of the particular variable across the control and treated samples. In **Figure 5.9 C**, for example, the variable trend plot shows that no change could be computed for the 327.214 *m/z* variable in the control samples, which could mean the absence of this variable in the non-treated samples. On the other hand, in the treated samples, the changes of the 327.214 *m/z* variable could be observed as infographically depicted by the trend plot (**Figure 5.9 C**), suggesting the presence of this ion (possible metabolite) in these samples and its trajectory changes over time. Thus, such an ion (variable) differentiates the control and treated samples.

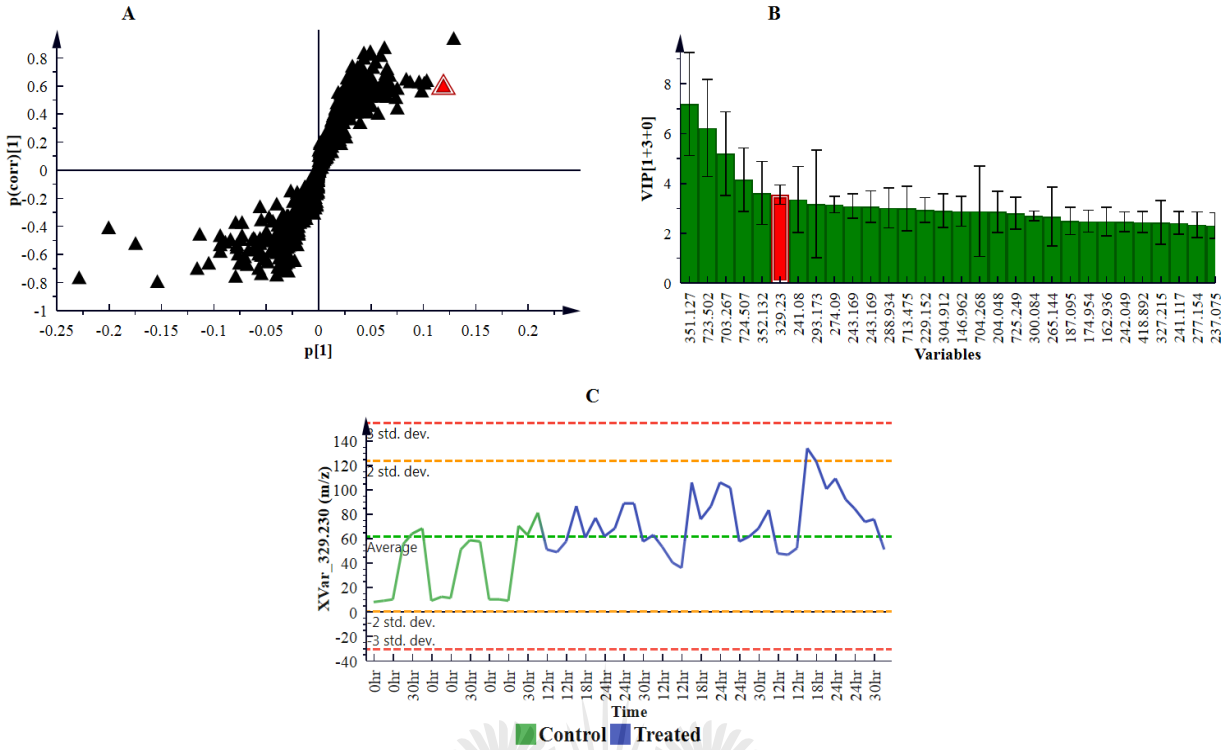


Figure 5.10: OPLS-DA modelling analyses of the LC-MS (ESI negative data) for <u>extracellular</u> extracts (excluding QCs). (A) An OPLS-DA loading S-plot displaying the discriminating features (ions) that explain the clustering (sample grouping) observed in the OPLS-DA scores plot with the features in the top right quadrant positively correlated to the treatment and those in the bottom left quadrant negatively correlated to the treatment. **(B)** A VIP plot summarising the importance of some of the variables in the projection of the model with the *m/z* values and jackknife confidence intervals reflecting the variable stability. A VIP value >1 indicates a significant variable in the complex analysis in comparing the difference between groups. **(C)** Representative variable trend plot exhibiting the changes of the selected variable across the control and treated samples.

Variables relating to the observed metabolic changes due to treatment with $LPS_{B.\ andr.}$, selected and validated with the aid of various chemometrics tools mentioned above, were further putatively identified (annotated at level 2 of the Metabolomic Data Standards Initiative (MSI-2)) (Sumner *et al.*, 2007), as described in **Chapter 3** (**section 3.6**). Metabolites shown in **Table 5.1** were annotated from both LC-MS ESI negative and positive data, and had a VIP score > 1. Fold changes presented in **Table 5.1** were obtained from the computed model of C0 h *vs* T 18 h, as this was the best time point representation of the overall metabolic changes. A similar table expressing the fold changes and *p*-values generated from the computed OPLS-DA models of other time points (*i.e.* C0 h *vs* 12-, 24- and 30 h), for intra- and extracellular data is included in the supplementary data file **Table S 5.1** and **S 5.2**, respectively.

Table 5.1: Annotated discriminatory metabolites from cell (intracellular) and medium (extracellular) extracts of LPS$_{B.\ andr.}$-treated *Sorghum bicolor* cultured cells. The summarised metabolites were annotated at MI-level 2 and had VIP score > 1. Fold changes were obtained from an OPLS-DA model of control (C0 h) *vs* treated 18 h. (*Data from 12, 24 and 30 h time points is presented as supplementary files*).

Metabolites	*m/z*	Rt (min)	Adduct	Ion mode	Formula	Intracellular			Extracellular			Class
						p-value	Fold change	Trend	*p*-value	Fold change	Trend	
Sorgoleone	359.2298	4.98	$[M+H]^+$	pos	$C_{22}H_{30}O_4$	0.048	3.0	Increase	8.78E-09	30.1	Increase	Allelochemical
L-Phenylalanine	164.0686	1.84	$[M-H]^-$	neg	$C_9H_{11}NO_2$	6.28E-06	1.4	Increase	3.07E-11	2.7	Increase	Amino acid
L-Tryptophan	203.0798	2.78	$[M-H]^-$	neg	$C_{11}H_{12}N_2O_2$	2.04E-06	1.4	Increase	0.001	1.4	Increase	Amino acid
15-Hydroxylinoleic acid	295.2253	14.29	$[M-H]^-$	neg	$C_{18}H_{32}O_3$	0.001	2.1	Increase	●	●	●	Fatty acid
Dihydroxy-octadecadienoic acid	311.2242	11.79	$[M-H]^-$	neg	$C_{18}H_{32}O_4$	1.28E-10	10.8	Increase	0.110	5.2	Increase	Fatty acid
9,10-Dihydroxy-12-octadecenoic acid	313.2354	12.67	$[M-H]^-$	neg	$C_{18}H_{34}O_4$	0.606	1.6	Increase	●	●	●	Fatty acid
9,10-Dihydroxystearic acid	315.2511	13.51	$[M-H]^-$	neg	$C_{18}H_{36}O_4$	2.37E-09	6.4	Increase	●	●	●	Fatty acid
Trihydroxy-octadecadienoic acid I	327.2149	9.72	$[M-H]^-$	neg	$C_{18}H_{32}O_5$	0.377	113	Increase	●	●	●	Fatty acid
Trihydroxy-octadecadienoic acid II	327.2135	11.05	$[M-H]^-$	neg	$C_{18}H_{32}O_5$	3.97E-13	356.7	Increase	0.000	38.8	Increase	Fatty acid
9,12,13-Trihydroxy-10-octadecenoic acid	329.2327	9.60	$[M-H]^-$	neg	$C_{18}H_{34}O_5$	0.000	1.8	Increase	1.13E-08	9.2	Increase	Fatty acid
16-Hydroxypalmitate	273.2553	13.65	$[M+H]^+$	pos	$C_{16}H_{32}O_3$	0.490	1.3	Increase	●	●	●	Fatty acid
Sophoraflavanone G	423.1821	4.42	$[M-H]^-$	neg	$C_{25}H_{28}O_6$	2.36E-05	0.8	Decrease	0.030	1.1	Increase	Flavonoid
Apigenin-8-*C*-glucoside (vitexin)	431.0974	5.58	$[M-H]^-$	neg	$C_{21}H_{20}O_{10}$	0.702	1.1	Increase	0.537	1.2	Increase	Flavonoid
Apigenin-6-C-xyloside-8-C-glucoside (vicenin-1)	565.1545	4.94	$[M+H]^+$	pos	$C_{26}H_{28}O_{14}$	0.064	0.6	Decrease	0.004	1.4	Increase	Flavonoid
Apigenin-6,8-di-C-glucoside (vicenin-2)	595.1687	4.77	$[M+H]^+$	pos	$C_{27}H_{30}O_{15}$	0.082	0.4	Decrease	0.240	2.1	Increase	Flavonoid
Apigenin 7,4'-dimethyl ether	316.1157	8.29	$[M+H\_NH_3]^+$	pos	$C_{17}H_{14}O_5$	0.000	0.5	Decrease	●	●	●	Flavonoid
3',4'5-Trihydroxy-3,7-dimethoxyflavone	367.0221	3.90	$[M-H]^-$	neg	$C_{17}H_{20}O_9$	0.032	0.8	Decrease	●	●	●	Flavonoid
4-Coumaroyl-3-hydroxyagmatine	291.1471	5.72	$[M-H]^-$	neg	$C_{14}H_{20}N_4O_3$	0.013	0.7	Decrease	0.001	2.5	Increase	HCA
4-Coumaroylquinic acid	337.1474	1.77	$[M-H]^-$	neg	$C_{16}H_{18}O_8$	7.19E-09	0.6	Decrease	0.010	1.1	Increase	HCA
Cinnamoylserotonin	351.1251	2.43	$[M-H\_HCOOH]^-$	neg	$C_{19}H_{18}N_2O_2$	7.77E-05	0.8	Decrease	0.000	1.2	Increase	HCA
Feruloylserotonin	351.1266	2.86	$[M-H]^-$	neg	$C_{20}H_{20}N_2O_4$	0.387	0.4	Decrease	0.771	1.2	Increase	HCA
Sinapaldehyde glucoside	369.1199	3.61	$[M-H]^-$	neg	$C_{17}H_{22}O_9$	0.525	0.9	Decrease	1.68E-06	2.7	Increase	HCA
1-O-Coumaroyl-beta-D-glucose	371.0957	4.94	$[M-H\_NaNa]^-$	neg	$C_{15}H_{18}O_8$	5.26E-11	0.6	Decrease	7.70E-06	2.1	Increase	HCA
Sinapoyl alcohol	209.0764	6.72	$[M-H]^-$	neg	$C_{11}H_{14}O_4$	1.55E-10	4.8	Increase	1.34E-05	1.8	Increase	HCA

Table 5.1 continued..

Dihydroconiferyl alcohol glucoside	413.1422	3.27	$[M+H\_HCOONa]^+$	pos	$C_{16}H_{24}O_8$	0.005	0.6	Decrease	●	●	●	HCA
Indole-3-butyric acid	272.0893	2.84	$[M+H\_HCOONa]^+$	pos	$C_{12}H_{13}NO_2$	1.62E-05	0.5	Decrease	●	●	●	Indole compound
N(6)-[(Indol-3-yl)acetyl]-L-lysine	304.1667	4.20	$[M+H]^+$	pos	$C_{16}H_{21}N_3O_3$	1.43E-07	5.8	Increase	●	●	●	Indole compound
Indole-3-acetyl-myo-inositol	353.1348	2.44	$[M-H\_NH_3]^-$	neg	$C_{16}H_{19}NO_7$	0.479	1.8	Increase	0.000	2.9	Increase	Indole compound
Indole-3-acetyl-beta-1-D-glucoside	382.1121	3.93	$[M-H\_HCOOH]^-$	neg	$C_{16}H_{19}NO_7$	0.075	0.7	Decrease	0.925	1.0	Increase	Indole compound
6-Hydroxy-indole-3-acetyl-valine	291.1294	3.89	$[M+H]^+$	pos	$C_{15}H_{17}N_2O_4$	0.174	0.5	Decrease	0.098	2.1	Increase	Indole compound
Traumatic acid	297.1291	3.90	$[M+H\_HCOONa]^+$	pos	$C_{12}H_{20}O_4$	0.027	4.4	Increase	●	●	●	Phytohormone
(9R,13R)-1a,1b-Dihomo-jasmonic acid	239.1638	12.19	$[M+H]^+$	pos	$C_{14}H_{22}O_3$	0.016	0.8	Decrease	●	●	●	Phytohormone
Zeatin-7-beta-D-glucoside	397.1826	6.73	$[M-H\_NH_3]^-$	neg	$C_{16}H_{23}N_5O_6$	0.216	0.8	Decrease	●	●	●	Phytohormone
Zeatin	220.1197	2.15	$[M+H]^+$	pos	$C_{10}H_{13}N_5O$	2.99E-05	0.5	Decrease	●	●	●	Phytohormone
Methyl jasmonate	247.1298	2.52	$[M+H\_Na]^+$	pos	$C_{13}H_{20}O_3$	1.21E-08	4.2	Increase	●	●	●	Phytohormone
Dihydrozeatin riboside	354.1769	6.20	$[M+H]^+$	pos	$C_{15}H_{23}N_5O_5$	0.000	0.2	Decrease	●	●	●	Phytohormone
Zeatin riboside	374.1463	5.39	$[M+H\_Na]^+$	pos	$C_{15}H_{21}N_5O_5$	0.000	0.3	Decrease	●	●	●	Phytohormone
Azelaic acid	187.0935	6.74	$[M-H]^-$	neg	$C_9H_{16}O_4$	7.24E-12	6.5	Increase	5.58E-07	1.9	Increase	Phytohormone
Abscisic acid	265.1552	3.35	$[M+H]^+$	pos	$C_{15}H_{20}O_4$	7.62E-08	3.5	Increase	4.39E-13	16.8	Increase	Phytohormone
Agmatine	173.0787	5.52	$[M-H\_NaNa]^-$	neg	$C_5H_{14}N_4$	0.000	1.4	Increase	●	●	●	Polyamine
Riboflavin	377.1476	4.49	$[M+H]^+$	pos	$C_{17}H_{20}N_4O_6$	0.050	0.7	Decrease	7.65E-08	5.9	Increase	Flavin

- ● : indicates the metabolite is absent in the particular extract.

HCA = hydroxycinnamic acid

Metabolite annotation was achieved with the aid from the Taverna workbench (www.taverna.org.uk), databases such as Dictionary of Natural Products (DNP) (dnp.chemnetbase.com), ChemSpider (www.chemspider.com), PubChem (www.pubchem.ncbi.nlm.nih.gov), PlantCyc (www.plantcyc.org), SorgCyc (www.sorgcyc.org) and KNApSAcK (http://kanaya.naist.jp/knapsack_jsp/top.htm) and available literature.

p-value refers to significance level of a metabolite. Fold change was calculated by dividing the average of the metabolite intensity in replicate samples of treated by the average of the metabolite intensity in replicate samples of control, a value ≥1 represents an increase (metabolite is higher in the treated samples than in the control) and value <1 represents a decrease (metabolite is higher in the control and treatment led to decrease in levels).

5.3. Metabolic changes induced by LPS*B. andr.* treatment in cultured *Sorghum bicolor* cells

The chemometrically selected variables were then annotated to structurally elucidated metabolites (**Table 5.1**), in order to gain biological insight into the changes occurring in the sorghum cell culture system, following LPS*B. andr.* treatment. The endometabolome (intracellular/fingerprint) and exometabolome (extracellular/footprint) of the cultured cells was characterised by metabolites associated with primary as well as secondary metabolism and of diverse biochemical functions in plant defence. As described previously (**Chapter 4**), the defence arsenal of sorghum is broad and diverse, involving metabolites of various chemical classes (Salzman *et al.*, 2005; Tugizimana *et al.*, 2014; Tugizimana, 2017). To have a comprehensive picture of metabolic reprogramming in sorghum cells induced by LPS treatment, relative quantification (based on fold changes) of the putatively identified metabolites was carried out, offering a measurable evaluation of metabolic changes.

Metabolic profiling of cultured cells revealed the intracellular induction of some metabolites of primary metabolism, L-phenylalanine and L-tryptophan, as well as excretion into the extracellular milieu (**Table 5.1**). As previously stated in **Chapter 4** (**section 4.4.1**) the role of amino acids in plant defence includes functioning as regulators and precursors in various secondary metabolic pathways involved in plant defence. L-Phenylalanine is a particularly important initiator/regulatory molecule of the phenylpropanoid pathway and is also involved in SA biosynthesis (Lattanzio *et al.*, 2006; Ayabe *et al.*, 2010; Tzin & Galili, 2010; Edwards, 2016; Ng *et al.*, 2016; Sun *et al.*, 2016). On the other hand, tryptophan is a major precursor in indolic secondary metabolite synthesis (Tzin & Galili, 2010; Zhao, 2012; Bottcher *et al.*, 2014; Pastorczyk & Bednarek, 2016). Moreover, the tryptophan metabolic pathway has also been reported to be involved in defence responses in cereal crops such as rice, through the production of serotonin and conjugates (see **Table 5.1**, on annotated serotonin conjugates in this study) (Ishihara *et al.*, 2008).

The intracellular up-regulation of phenylalanine and tryptophan (fold change >1) across the time points (**Table 5.1, Table S 5.1** and **Figure 5.11**) could be as a result of continuous requirement for the precursors in the synthesis of phenylalanine-derived and tryptophan-derived metabolites, respectively. The presence of phenylalanine and some phenylpropanoids (and other related secondary metabolites) in the cell extracts, indicates that these metabolic

pathways were activated by the cells in response to 'non-self' perception of LPS as a MAMP. Similarly, the presence of tryptophan (a major precursor) and tryptophan-derived metabolites such as indoles and serotonin conjugates, is also indicative of the active involvement of these pathways in immune responses, following LPS treatment.

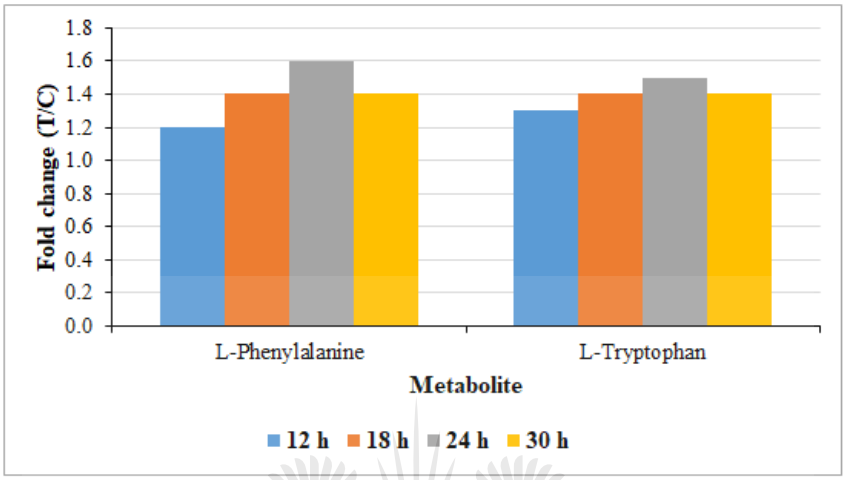


Figure 5.11: Relative quantification of amino acids annotated in intracellular extracts, induced by LPS treatment of sorghum cells. The graph shows the relative levels of each metabolite across the time points, expressed as fold changes, computed from treated against control (C0 h) *i.e.* T/C, where fold change > 1 represents significant accumulation.

The *B. andropogonis* derived elicitor, LPS, triggered significant changes in lipidome components *i.e.* various fatty acid pathways were activated which led to subsequent significant accumulation of downstream products (mostly as hydroxy fatty acids). As infographically shown in **Figure 5.12 A** and **B**, these hydroxy fatty acids accumulated in the intracellular milieu of treated cells to varying degrees, with levels fluctuating across the time points (see **Table 5.1** and **S 5.1** for fold changes of each metabolite at different time points). Assessing the quality of the endo- and exolipidome, some fatty acids and derivatives were detected in both intra- and extracellular extracts, whereas others were exclusive to the endometabolome (15-hydroxylinoleic acid, 9,10-dihydroxy-12-octadecenoic acid, 9,10-dihydroxystearic acid, trihydroxy-octadecadienoic acid I and 16-hydroxypalmitate) (**Table 5.1**). Fatty acids and derivatives thereof are crucial in basal immunity and gene-mediated resistance. They also take part in inducing systemic acquired resistance. Fatty acids, such as those putatively identified in this study, have been linked to plant defence responses as enhancers of structural defence (cell membrane and cell wall), antimicrobial compounds, key players in plant defence signalling pathways (*e.g.* oxylipins) and to play a role during production of JA, an important mediator of plant defence (Hou, 2008; Hamberg & Olsson, 2011; Pohl *et al.*, 2011; Hamberg *et al.*, 2003;

Walley *et al.*, 2013; Okazaki & Saito, 2014; Gauthier *et al.*, 2015; Lim *et al.*, 2017). The functions of some fatty acids in this present study, also identified in sorghum plants in response to the live *B. andropogonis* pathogen, are highlighted in the previous chapter (**Chapter 4; section 4.4.2**).

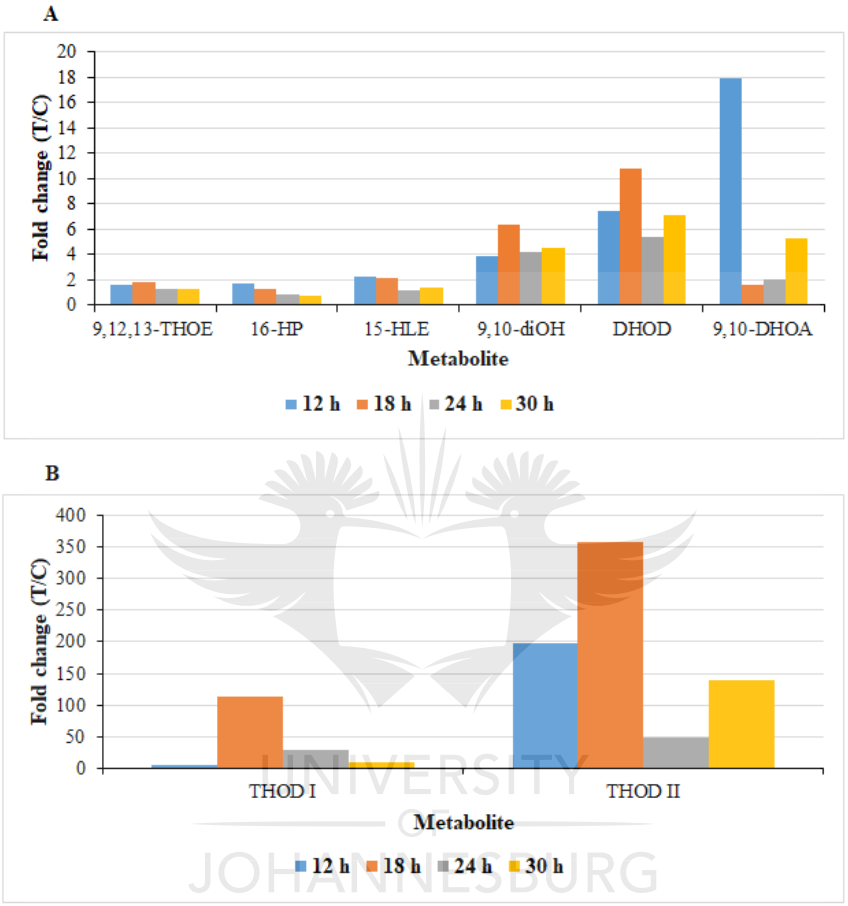
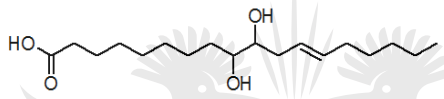
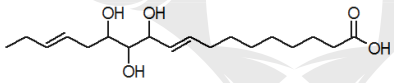
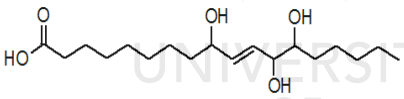


Figure 5.12: Relative quantification of fatty acids annotated in intracellular extracts, induced by LPS treatment of sorghum cells. The graph shows the relative levels of each metabolite across the time points, expressed as fold changes, and computed from treated against control (C0 h) *i.e.* T/C, where fold change ≥1 represents significant accumulation. Graphical representation of the annotated fatty acids is divided into two graphs; **A** for reasonably high fold changes and **B** for immensely high fold changes. 9,12,13-THOE = 9,12,13-trihydroxy-10-octadecenoic acid; 15-HLE = 15-hydroxylinoleic acid; DHOD = dihydroxy-octadecadienoic acid; 16-HP = 16-hydroxypalmitate; 9,10-diOH = 9,10-dihydroxystearic acid; 9,10-DHOA = 9,10-dihydroxy-12-octadecenoic acid; THOD I = trihydroxy-octadecadienoic acid I; THOD II = trihydroxy-octadecadienoic acid II.

Our results show significant accumulation of trihydroxy oxylipins, trihydroxy-octadecadienoic acid I, trihydroxy-octadecadienoic acid II and 9,12,13-trihydroxy-10-octadecenoic acid and the dihydroxy-oxylipin, 9,10-dihydroxy-12-octadecenoic acid (see **Table 5.2** for the oxylipin structures) in LPS-treated cells. Based on correlative data and experimental work, several trihydroxy-oxylipins have been shown to exhibit antimicrobial activity and establish resistance towards fungal and some bacterial pathogens, and to orchestrate defence responses. For such

metabolites to be significantly effective in inhibiting pathogen growth *in planta*, they should be available in adequate concentrations. Previously, it has been demonstrated that trihydroxy oxylipins have an ability to induce defence responses and contribute to resistance in cereal plants such as barley against powdery mildew (Göbel *et al.*, 2001, 2002; Walters *et al.*, 2006; Hamberg & Olsson, 2011), and rice against rice blast disease (Hou & Forman III, 2000; Göbel *et al.*, 2002).

Table 5.2: Structural representations of some lipidome components, oxylipins annotated as constituents of the endometabolome (intracellular) and/or exometabolome (extracellular) of LPS-treated cultured *Sorghum bicolor* cells. These fatty acids significantly accumulated following LPS$_{B.\ andr.}$ elicitation, suggesting important functions in the defence response of cultured cells.

Oxylipin	Stucture	Molecular formula	Molecular weight	Source
9,10-Dihydroxy-12-octadecenoic acid		$C_{18}H_{34}O_4$	314.46	PubChem
Trihydroxy-octadecadienoic acid I/II		$C_{18}H_{32}O_5$	328.44	
9,12,13-Trihydroxy-10-octadecenoic acid		$C_{18}H_{34}O_5$	330.46	

The oxylipin of interest is the trihydroxy-octadecadienoic acid II, which was positively correlated to the treatment, and exhibited an immense accumulation in the cells of 356.7-fold at the 18 h time point (**Table 5.1**). An unsupervised colour-coded PCA score plot (**Figure 5.13**) revealed the presence of this metabolite only in treated cells (intracellular extracts) and absent in the control (non-treated) samples (as also seen variable trend plot, **Figure 5.9 C**), implying a *de novo* biosynthesis of the metabolite as induced by LPS treatment. This finding, thus, suggests a defence-related role of this fatty acid as a phytoalexin. To the best of our knowledge no studies have been reported on the secretion of this metabolite into the cell periphery but our data indicates a significant accumulation in the medium of 38.8-fold at the 18 h time point. Trihydroxy-octadecadienoic acids such as 9,12,13-trihydroxy-10,15-octadecadienoic acid have been shown to inhibit fungal growth (Hou & Forman III, 2000; Göbel *et al.*, 2001, 2002; Prost, 2005; Walters *et al.*, 2006; Abad-Garcı´a *et al.*, 2008) and to inhibit bacterial pathogens

(Walters *et al.*, 2006). Another oxylipin, 9,12,13-trihydroxy-10-octadecenoic acid was demonstrated to inhibit fungal pathogens and confer resistance towards a wide range of fungal pathogens (Hamberg, 1997, 1999; Göbel *et al.*, 2002; Prost, 2005; Walters *et al.*, 2006; Aghofack-Nguemezi *et al.*, 2011; Hamberg & Olsson, 2011) and also bacterial pathogens (Göbel *et al.*, 2002; Vicente *et al.*, 2012). In a study by Prost (2005), the dihydroxy oxylipin, 9,10-dihydroxy-12-octadecenoic acid was identified as an antimicrobial compound. However, the mechanisms by which these oxylipins inhibit microbial growth through antimicrobial activity and establish resistance, is still largely undefined (Göbel *et al.*, 2002). The present work, thus, demonstrates the importance of the oxylipin and fatty acid pathways in defence responses in sorghum cells, based on significant accumulation. This observation is also supported by the accumulation of the fatty acid-derived jasmonate oxylipins (*e.g.* MeJA) as defence signalling molecules in treated sorghum cells.

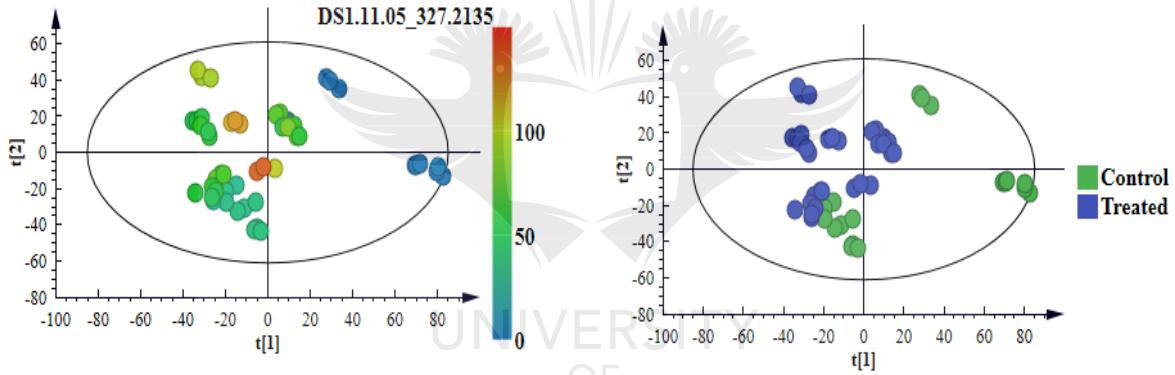


Figure 5.13: PC analyses of LC-MS (ESI negative) data of intracellular extracts from Sorghum cells. (A) An unsupervised colour-coded PCA score plot displaying the presence/absence and intensity of the trihydroxy-octadecadienoic acid II phytoalexin across intracellular samples**. (B)** A similar corresponding PCA score plot, coloured coded based on condition (treated/control) to assist in indicating if samples belong to the control or treated group. The absence of the metabolite in non-treated (control) samples and presence in the treated samples indicate LPS-induced *de novo* biosynthesis.

As summarised in **Table 5.1** LPS triggered an alteration of several plant hormones (jasmonates, zeatins, traumatic-, azelaic- and abscisic acid) (also see **Figure 5.14**). The derivative of JA, MeJA, and traumatic acid accumulated exclusively in the intracellular milieu to significantly high levels (**Table 5.1**). MeJA, a well-known volatile fatty acid-derived plant stress hormone, is involved in the activation of defence mechanisms such as programmed cell death, ROS production, lignin formation and wax layers deposition in plant tissues (Ali *et al.*, 2007; Zhang & Xing, 2008; Taheri & Tarighi, 2010; Petrussa *et al.*, 2013; Gauthier *et al.*, 2015). This plant hormone observed to generally increase across the time points (**Figure 5.14**), has been reported

as a signalling molecule in elicitor-induced plant cell cultures and plant tissue, initiating secondary metabolite accumulation (Sudha & Ravishankar, 2002; Zhao *et al.*, 2005; Petrussa *et al.*, 2013). Studies on exogenous application of the hormone, revealed an association with cellular metabolome reprogramming – stimulation of the phenylpropanoid, flavonoid, fatty acid and other secondary metabolic pathways (Sudha & Ravishankar, 2002; Salzman *et al.*, 2005; Abdel-Farid *et al.*, 2009; Liu *et al.*, 2010; Gauthier *et al.*, 2015; Tugizimana *et al.*, 2015; Ramirez-Estrada *et al.*, 2016). Together with ethylene, JA and derivatives are regarded as the main role players in induced systemic research (ISR).

Moreover, a study on transcriptional profiling of genes induced by SA and MeJA in sorghum revealed that these hormones coordinately induced genes encoding various enzymes catalysing the biosynthesis of anthocyanins, phytoalexins, lignin and other defence-related secondary metabolites of the phenylpropanoid pathway. Functionally important enzymes of this pathway such as phenylalanine ammonia-lyase, cinnamate-4-hydroxylase, cinnamyl alcohol dehydrogenase, cinnamoyl-CoA reductase, chalcone synthase and chalcone-flavanone isomerase, amongst others, required in the synthesis of defence secondary metabolites, were found to be induced by the phytohormones SA and MeJA – in sorghum (Salzman *et al.*, 2005; Poloni & Schirawski, 2014). Additionally, some evidence presented, shows that exogenous application of MeJA enhances resistance towards necrotrophic pathogens (Antico *et al.*, 2012; Gauthier *et al.*, 2015; Shigenaga & Argueso, 2016). Another jasmonate – (9R,13R)-1a,1b-dihomo-jasmonic acid, was identified in intra- and extracellular extracts, however, the hormone accumulated in low levels. This jasmonate was reported to accumulate in *Fusarium graminearum*-treated barley (Guptha, 2016) and LPS-treated *Arabidopsis* cells (Finnegan, 2012). This significant MeJA accumulation following LPS treatment points to a crucial role of jasmonates defence responses in cultured sorghum cells.

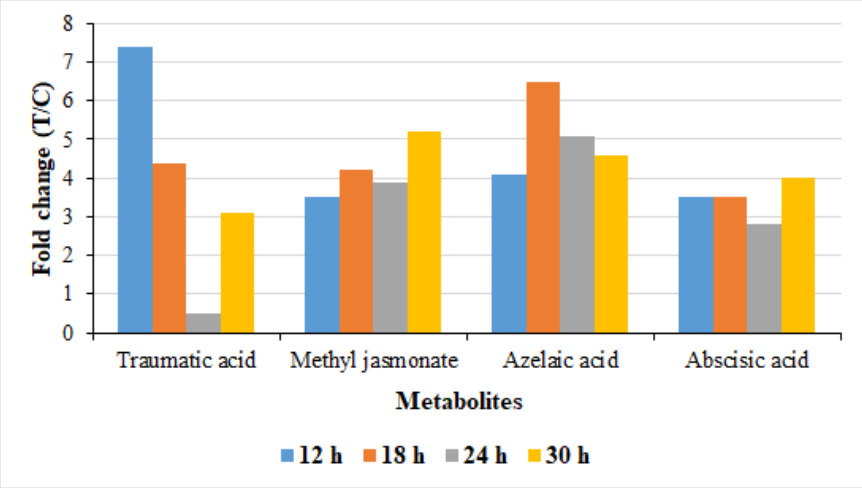


Figure 5.14: Relative quantification of significantly accumulating plant hormones annotated in intracellular extracts, induced by LPS treatment. The graph shows the relative levels of each metabolite across different time points, expressed as fold changes, computed from treated against control (C0 h) *i.e.* T/C, where fold change ≥1 represents significant accumulation.

Another fatty acid-derived phytohormone, traumatic acid, exhibited significant accumulation in cells (**Table 5.1**). Traumatic acid, which displayed very high levels in at the early stages (12-18 h) of the treatment (**Figure 5.14**), is generally known as a wound hormone due to high accumulation around wounded areas (Farmer, 1994; Pietryczuk & Czerpak, 2012; Jabłońska-Trypuć *et al.*, 2016). Synthesis of traumatic acid is commonly associated with abiotic factors such as extreme temperatures (low/high), osmotic shock, wounding and UV damage (Sivasankar *et al.*, 2000; Pietryczuk & Czerpak, 2012). However, the hormone was also identified as a resistance inducing metabolite in barley, conferring resistance to *F. graminearum* (Chamarthi *et al.*, 2014). The detected levels of the hormone suggest a defence role in LPS-treated cultured sorghum cells. Other identified hormones included zeatins (also exclusive to the endometabolome). Relative quantitative analysis revealed low levels thereof in the endometabolome (**Table 5.1** and **S 5.1**). Although the zeatin-hormones were detected in low levels (shown by the decrease in trend), the involvement of this class of hormones in mediating defence responses in sorghum cells cannot be ruled out. Some roles of zeatin-hormones in plant defence have been highlighted in the previous chapter (**Chapter 4**; **section 4.4.3**). In addition, the involvement of zeatin-cytokinins in plant–environment interactions, has been reported (Veselova *et al.*, 2006; Schäfer *et al.*, 2015).

The hormones azelaic acid and abscisic acid were identified in increased levels in both endo- and exometabolomes (**Table 5.1**). As previously stated in **Chapter 4** (**section 4.4.2**), ROS – also accumulating in response to biotic stress (*e.g.* pathogen challenge) (Eckardt, 2008; Gao *et*

al., 2015), can trigger the cleavage of fatty acids yielding products such as azelaic acid (Walley *et al.*, 2013; Barbaglia & Hoffmann-Benning, 2016; Shine *et al.*, 2018), a signalling molecule also associated with SAR (Manosalva *et al.*, 2010; Kliebenstein, 2012; Okazaki & Saito, 2014; Barbaglia & Hoffmann-Benning, 2016; Shine *et al.*, 2018). It has been previously proposed that the induction of SAR by azelaic acid is through priming of plants to accumulate enhanced SA levels and SA-facilitated responses in pathogen infection. However, recent findings suggest the induction of SAR by the metabolite through stimulating glycerol-3-phoshphate (G3P) accumulation (Kachroo & Robin, 2013; Gao *et al.*, 2014, 2015; Tugizimana *et al.*, 2014; Wittek *et al.*, 2014; Lim *et al.*, 2017). In plants, following pathogen infection, azelaic acid – as one of the signalling molecules, accumulates in petiole exudates and a small fraction translocates to distal tissue (in its free form or derivatised form) (Shah, 2009; Gao *et al.*, 2014; Barbaglia & Hoffmann-Benning, 2016; Shine *et al.*, 2018).

Abscisic acid is generally known to largely regulate defence responses to abiotic stress. However, the complex role of the hormone in plant immunity is continuously being uncovered, with recently emerged insights into abscisic acid's role in plant–pathogen interactions as a positive or negative defence response regulator, depending on the phase/time of infection and nature of the pathogen (Bari & Jones, 2009; Bollina *et al.*, 2011; Atkinson & Urwin, 2012; Balmer *et al.*, 2013; Denancé *et al.*, 2013; Liu *et al.*, 2015; Shigenaga & Argueso, 2016). Moreover, in stress responses, the hormone is now being viewed as a global regulator due to the ability to prioritise plant defence responses to a more grievous threat (either biotic or abiotic) (Atkinson & Urwin, 2012). Defence mechanisms such as stomatal closure, induced by abscisic acid signalling in order to inhibit bacterial invasion, have been reported (Bari & Jones, 2009; Atkinson & Urwin, 2012; Denancé *et al.*, 2013; Vidhyasekaran, 2015; Barbaglia & Hoffmann-Benning, 2016). In some plant cell cultures, abscisic acid has also been reported in the regulation of secondary metabolite biosynthesis, for example in *Catharanthus roseus*, the hormone stimulated indole alkaloids accumulation (Zhao *et al.*, 2005). Notably, the hormone was shown to positively regulate systemic resistance to pathogens, such as *Pythium irregular* and *Alternaria brassicicola* in early phase of infection, with mutants deficient and insensitive to the hormone displaying pathogen susceptibility (Bari & Jones, 2009; Atkinson & Urwin, 2012; Balmer *et al.*, 2013; Denancé *et al.*, 2013) and inhibiting *C. graminicola* fungal growth in maize (Balmer *et al.*, 2013). Similarly, the levels of abscisic acid detected in intra- and extracellular extracts (in this study) can be linked to resistance/launch of defence responses in cultured sorghum cells triggered by LPS perception. In conjunction with other studies, the

results of this study also suggest that diverse signalling molecules, particularly those that were found to accumulate significantly as biomarkers, mediate defence responses in sorghum cells.

Elicitation of cultured cells with LPS induced alterations in the phenylpropanoid and flavonoid metabolic pathways. Relative quantification revealed that most of the metabolites arising from these pathways were mostly associated with a decrease in levels (fold change <1) at most of the time points in the intracellular milieu (except for sinapoyl alcohol, displaying of fold change ≥1 across all time points in both intra- and extracellular milieu, **Table 5.1** and **S 5.1-2**). This can be seen in **Figure 5.15 A** for flavonoids and **Figure 5.16 A** for hydroxycinnamic acids. Out of the fourteen phenolic compounds annotated, only sinapoyl alcohol was positively correlated (data not shown) to the LPS treatment *i.e.* the other thirteen compounds were located/extracted from the bottom left quadrant of the OPLS-DA derived S-plot (see **Figure 5.9 A** and **S 5.7 A**). In contrast, the flavonoids and hydroxycinnamic acids displayed an increase in the extracellular milieu (fold change >1) as seen in **Figure 5.15 B** and **Figure 5.16 B**, respectively. Previous studies have shown that LPS treatment leads to induction/higher levels of phenolics in tobacco cells (Mhlongo *et al.*, 2016) and Arabidopsis cells (Finnegan *et al.*, 2016). However, in this study the opposite was observed. According to Mhlongo *et al.* (2016) and Gamir *et al.* (2014) the perceived stimulus and the system determines the pool of induced metabolites.

Decrease in intracellular levels of secondary metabolites, accompanied by an increase extracellularly, may be due to the active translocation/secretion to the outside of the cell into the culture medium, in this case (and into the apoplast/cell periphery in the tissue environment). Distribution of defence-related metabolites to the sites of early pathogen infection is crucial for the restriction of pathogen penetration and proliferation (Bednarek *et al.*, 2009), and a study on *Arabidopsis thaliana* revealed the secretion of indolic glucosinolates into the cell periphery upon MAMP-triggered immunity (Finnegan *et al.*, 2016). Another possible explanation for the low intracellular levels of the phenylpropanoids and flavonoids in the cultured sorghum cells is the regulation of secondary metabolite levels by the cells, to avoid toxicity to the producing plant cell. At certain levels some of the secondary metabolites become toxic to the producing cells, so the cells regulate levels in order to bring balance to the cell, thus the compounds can be stored in other forms or broken down or translocated (Goossens *et al.*, 2003). Regulatory mechanisms by plants include transportation to the apoplast (*via* vesicles, simple diffusion, and transporter-mediated membrane transport) or specific organelles such as the vacuole, or other

self-tolerance mechanisms (Shitan, 2016). Additionally, the decrease in intracellular phenolics levels could also be due polymerisation of metabolites that act as monomers (*e.g.* 4-coumaroyl-3-hydroxyagmatine, feruloylserotonin, sinapaldehyde glucoside and dihydroconiferyl alcohol glucoside) for polymers such as lignin associated with cell wall reinforcement or due to transformation/conversion into other defence-related metabolites (Gunnaiah *et al.*, 2012; Chamarthi *et al.*, 2014; Gunnaiah & Kushalappa, 2014; Yogendra *et al.*, 2014; Gauthier *et al.*, 2015; Lowe *et al.*, 2015; Cuperlovic-Culf *et al.*, 2016).

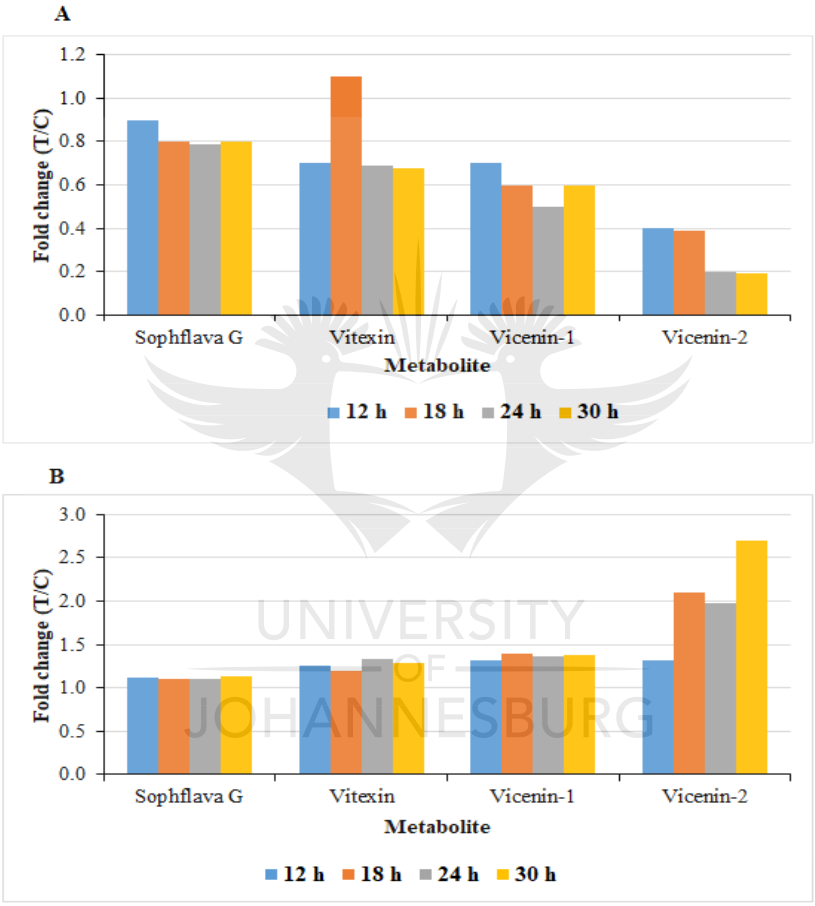


Figure 5.15: Relative quantification of some flavonoids annotated in intracellular (A) and extracellular (B) extracts, induced by LPS treatment. The relative levels of each metabolite are expressed in fold changes, computed from treated against control (C0 h) *i.e.* T/C, where fold change ≥1 represents significant accumulation. Sophflava G = sophoraflavanone G; vicenin-1 = apigenin 6-C-xyloside-8-C-glucoside; vicenin-2 = apigenin-6,8-di-C-glucoside; vitexin = apigenin-8-*C*-glucoside.

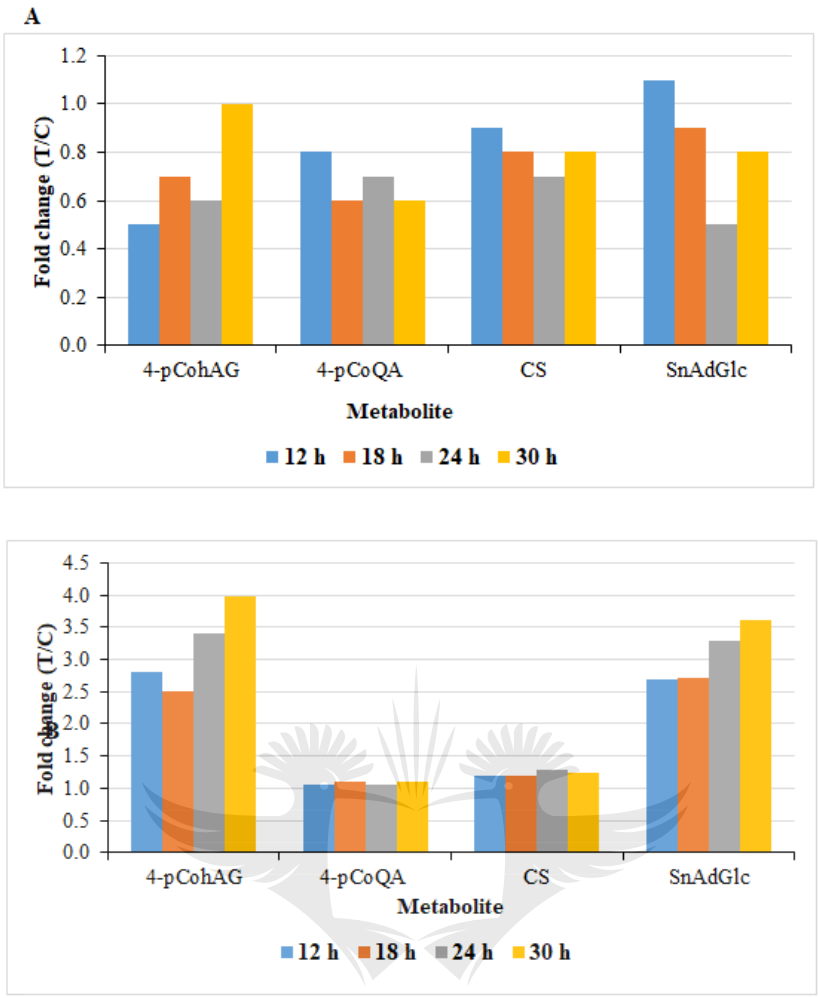


Figure 5.16: Relative quantification of some hydroxycinnamic acids annotated in <u>intracellular</u> (A) and <u>extracellular</u> (B) extracts, induced by LPS treatment. The relative levels of each metabolite are expressed in fold changes, computed from treated against control (C0 h) *i.e.* T/C, where fold change ≥1 represents significant accumulation. 4-*p*CohAG = 4-coumaroyl-3-hydroxyagmatine; 4-*p*CoQA = 4-coumaroylquinic acid; CS = cinnamoylserotonin; SnAdGlc = sinapaldehyde glucoside.

A focus on the identified flavonoids indicates that flavones (apigenin derivatives) was the dominant subgroup (**Table 5.1**). The biological functions of metabolites belonging to this subgroup in plant defence, such as exhibiting antimicrobial properties towards various pathogens, have been described in a number plants (Mohamed & El-hadidy, 2008; Balmer *et al.*, 2013; Mierziak *et al.*, 2014; Refaat *et al.*, 2015), and in sorghum plant tissue towards *B. andropogonis* as reported in **Chapter 4**; **section 4.4.4.1.** The tetrahydroxyflavanone, sophoraflavanone G, also identified amongst the flavonoids, possesses antibacterial properties. The mechanism of action of this flavonoid includes the alteration of bacterial membrane fluidity (Naoumkina *et al.*, 2010; Kumar & Pandey, 2013). The detection of sophoraflavanone G in plant extracts (**Chapter 4**; **Table 4.2**) and cell extracts (**Table 5.1**) can be similarly linked to a defence-related role. The detection of the metabolite following LPS elicitation suggests

MAMP-triggered accumulation. The mechanisms of action of some flavonoids in immune responses have been listed in the previous chapter (**Chapter 4**), however, detailed molecular mechanisms of this class of metabolites in sorghum–*B. andropogonis* and sorghum–LPS interactions still requires further investigation.

The roles of the identified hydroxycinnamic acids (HCAs) in plant defence include: cell wall strengthening, precursors to defence metabolites and as antimicrobial compounds (Wang *et al.*, 2013; Mhlongo *et al.*, 2014, 2016; Pushpa *et al.*, 2014; Tugizimana *et al.*, 2014; Lowe *et al.*, 2015). As previously described in **Chapter 4**, sinapoyl alcohol, a syringyl lignin precursor, is an important metabolite in structural/mechanical defence (cell wall enhancement). This is through lignification *i.e.* polymerisation of monolignols (precursors of lignin), resulting in reinforcement of the cell wall to become more resistant to pathogen-derived degrading enzymes and penetration by mycotoxins. In general, increased accumulation of this precursor associated with lignin deposition has been reported following pathogen challenge. Treatment with LPS resulted in high accumulation of sinapoyl alcohol (for example, 4.8-fold intracellular and 1.8-fold extracellular, at the 18 h time point; **Table 5.1**), thus, suggesting the importance of the metabolite in the activation of structural defences in the cultured cells (Wang *et al.*, 2013; Gunnaiah & Kushalappa, 2014; Pushpa *et al.*, 2014; Gauthier *et al.*, 2015).

Other metabolites identified in this study also reported to be deployed in cell wall strengthening include 4-coumaroyl-3-hydroxyagmatine, feruloylserotonin, sinapaldehyde glucoside, cinnamoylserotonin and dihydroconiferyl alcohol glucoside (a guacoyl lignin monomer glucoside). In other studies these metabolites have also been identified as resistance-related compounds (Gunnaiah *et al.*, 2012; Chamarthi *et al.*, 2014; Gunnaiah & Kushalappa, 2014; Yogendra *et al.*, 2014; Gauthier *et al.*, 2015; Cuperlovic-Culf *et al.*, 2016). Additionally, 4-coumaroylquinic acid has been shown to confer resistance against a range of pathogens (Gunnaiah *et al.*, 2012; Yogendra *et al.*, 2014; Cuperlovic-Culf *et al.*, 2016). Agmatine, which can conjugate with HCAs to yield HCA-amides, and important metabolites in cell wall strengthening and as phytoalexins, displayed an increase and was found exclusively in the endometabolome (Gunnaiah *et al.*, 2012; Pushpa *et al.*, 2014; Yogendra *et al.*, 2014; Cuperlovic-Culf *et al.*, 2016).

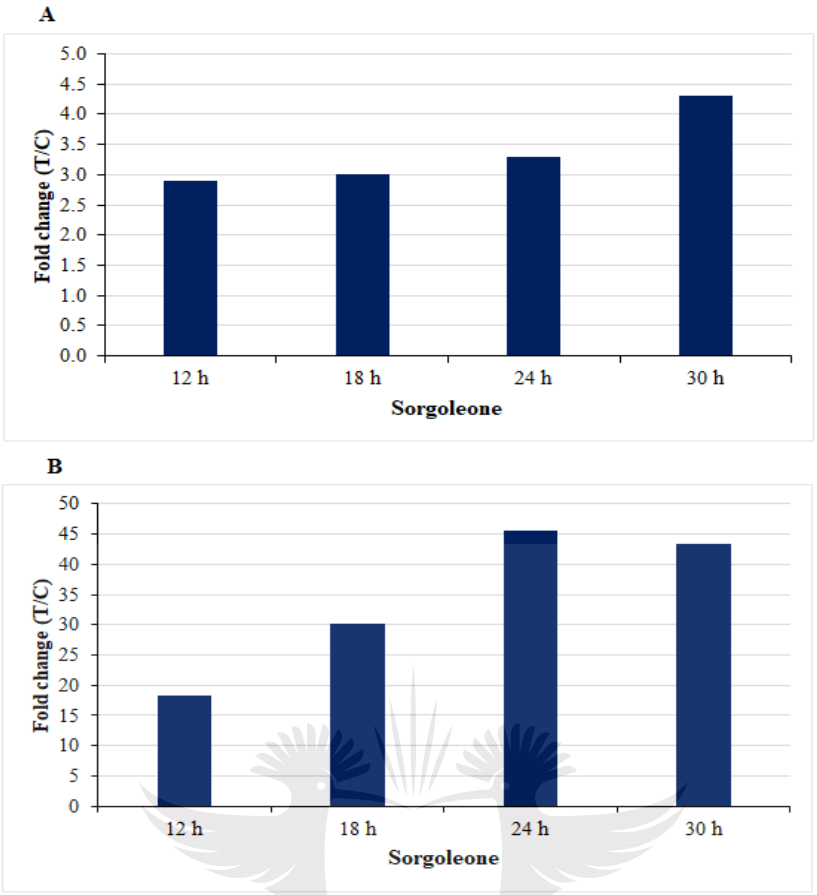


Figure 5.17: Relative quantification of sorgoleone across the time points, annotated in <u>intracellular</u> (A) and <u>extracellular</u> (B) extracts, induced by LPS treatment of sorghum cells. The graph shows the relative levels of each metabolite across different time points, expressed as fold changes, and computed from treated against control (C0 h) *i.e.* T/C, where fold change > 1 represents significant accumulation.

An interesting metabolite, sorgoleone (2-hydroxy-5-methoxy-3-[(Z,Z)-8',11',14'-pentadecatriene]-p-benzoquinone), significantly accumulated in the intracellular milieu and was also detected in the extracellular milieu in relatively higher levels (**Table 5.1**), with levels generally showing an increase over time (12-30 h) intracellularly (**Figure 5.17 A**) and extracellularly (**Figure 5.17 B**), following LPS treatment. Sorgoleone, an allelochemical, is one of the main constituents of the hydrophobic root exudate of sorghum, synthesised in compartments of root hairs (specialised root hair cells). Identification of sorgoleone as a biomarker in cultured sorghum cells is quite interesting and may be related to the undifferentiated state of the cells. The metabolite, which is exclusive to sorghum species, exhibits herbicidal activity – suppressing other plant species (especially small weeds) growing in the vicinity (Dayan, 2006; Cook *et al.*, 2010; Dayan *et al.*, 2010; Yoneyama & Natsume, 2010; Uddin *et al.*, 2014; Lim *et al.*, 2017). In addition, some antifungal activities of the metabolite and production due to pathogen infection, have been reported (Dayan, 2006). The

biosynthetic pathway of the allelochemical is a convergence of the fatty acid and polyketide synthase pathways (Dayan, 2006; Cook *et al.*, 2010; Dayan *et al.*, 2010). Significant accumulation of sorgoleone in the intra- and extracellular milieu, for example 3.0-fold and 30.1-fold respectively as seen at the 18 h time point in **Table 5.1**, following LPS treatment, also suggest a probable antimicrobial activity of the metabolite, however, more research needs to be done in this regard.

The metabolic reprograming in sorghum cells as a result of LPS treatment also involved some alterations in flavin and indole metabolism. The metabolite profiles of treated cells were characterised by down-regulation and up-regulation of riboflavin (vitamin B_2) in the intra- (0.7-fold) and extracellular (5.9-fold) milieu, respectively (**Table 5.1**). This is indicative of the secretion of the metabolite into the latter. Riboflavin has been identified as a defence response/systemic resistance inducing metabolite in various plant species, against bacterial, fungal and viral pathogens (Aver'yanov *et al.*, 2000; Dong & Beer, 2000; Taheri & Tarighi, 2010, 2011; Nie & Xu, 2016). In the monocotyledonous crop - rice, the metabolite was shown to induce defence responses against *Rhizoctonia solani* and *Pyricularia oryzae* (Aver'yanov *et al.*, 2000; Taheri & Tarighi, 2010, 2011). A study by Taheri & Tarighi, (2010) on rice also highlighted the major role of the octadecanoid pathway in riboflavin-induced resistance and basal resistance, together with the link between riboflavin accumulation and increased lignification. The data obtained in this study, therefore, suggests a role of flavin metabolism in inducing or regulating defence responses in sorghum cells.

Indolic derivatives (mostly indole acetyl derivatives) accumulated to varying levels, in both the intra- and extracellular milieus (**Table 5.1**). A number of plant species and plant cell cultures have been reported to accumulate indolic derivatives as phytoanticipins, phytoalexins, precursors or as signalling molecules in response to pathogen and abiotic stress (Ishihara *et al.*, 2008; Shah, 2009; Balmer *et al.*, 2013; Denancé *et al.*, 2013; Bottcher *et al.*, 2014; Finnegan *et al.*, 2016; Pastorczyk & Bednarek, 2016; Wouters *et al.*, 2016). Indole-3-acetyl-myo-inositol, significantly detected in both the intra- and extracellular milieus, has been linked to plant resistance in barley (Bollina *et al.*, 2011; Cuperlovic-Culf *et al.*, 2016). The indole derivatives, particularly those that accumulated to significant levels following treatment, suggest defence-related roles in sorghum cells. Moreover, the detection of both tryptophan and indole-containing metabolites as discriminant ions provides an insight into the activation of defences related to tryptophan metabolism, in response to LPS treatment.

Based on structural and compositional analysis, it can be concluded that LPS isolated from *B. andropogonis* (M_r > 7.5 kDa) is comprised of all the three main structural components; O-antigen/O-chain covalently linked *via* a core oligosaccharide to the Lipid A component (glycolipid moiety). The presence of the O-chain therefore denotes the isolated MAMP as a 'smooth'-type LPS (as defined in **Chapter 2**; **section 2.3.3**). The metabolic reprogramming in sorghum cells following $LPS_{B.\ andr.}$ treatment, thus, marked the onset of defence responses mediated by a range of plant hormones linked in a complex network, and triggering accumulation of defence-related metabolites originating from various metabolic pathways (Pieterse *et al.*, 2009; Atkinson & Urwin, 2012; Denancé *et al.*, 2013; Vidhyasekaran, 2015). The presence of metabolites in the medium in elevated levels following LPS treatment, strongly suggests a secretion/translocation from intracellular compartments, resembling secretion/translocation into the apoplast/cell periphery in the plant tissue environment, as revealed by the experimental data of the current study (**Table 5.1, S 5.1-2**). This activity may be linked to defence-related functions by these particular metabolites. Shitan, (2016) highlighted the translocation of various classes of metabolites (such as monolignols important for lignification, phenylpropanoids and flavonoids) to the apoplast for purposes such as defence. In summary, the picture depicted by this study implies that $LPS_{B.\ andr.}$ is one of the instrumental factors (MAMPs) of *B. andropogonis* in triggering the defence-related metabolomic reprogramming in sorghum.

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Chapter 5: Supplementary material

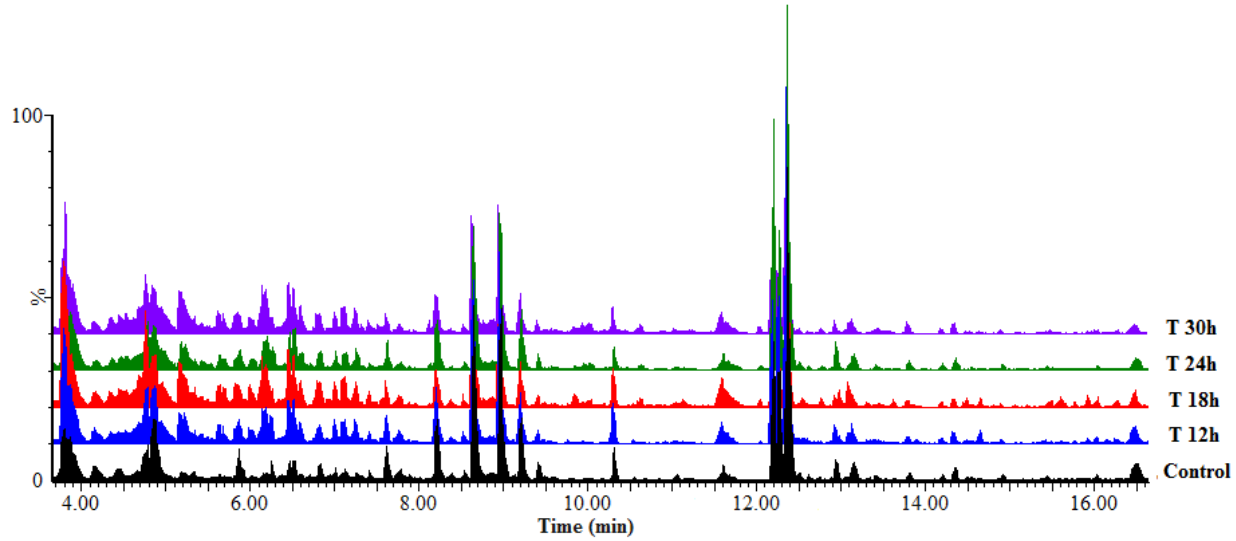

Figure S 5.1: UHPLC-MS BPI chromatograms (ESI positive) of methanolic <u>intracellular</u> extracts of sorghum cells treated with LPS*B. andr.* The chromatograms of a control (non-treated 0 h) *vs* treated samples (12-30 h) display variation related to treatment- and time-related metabolic changes occurring in the cells due to LPS treatment.

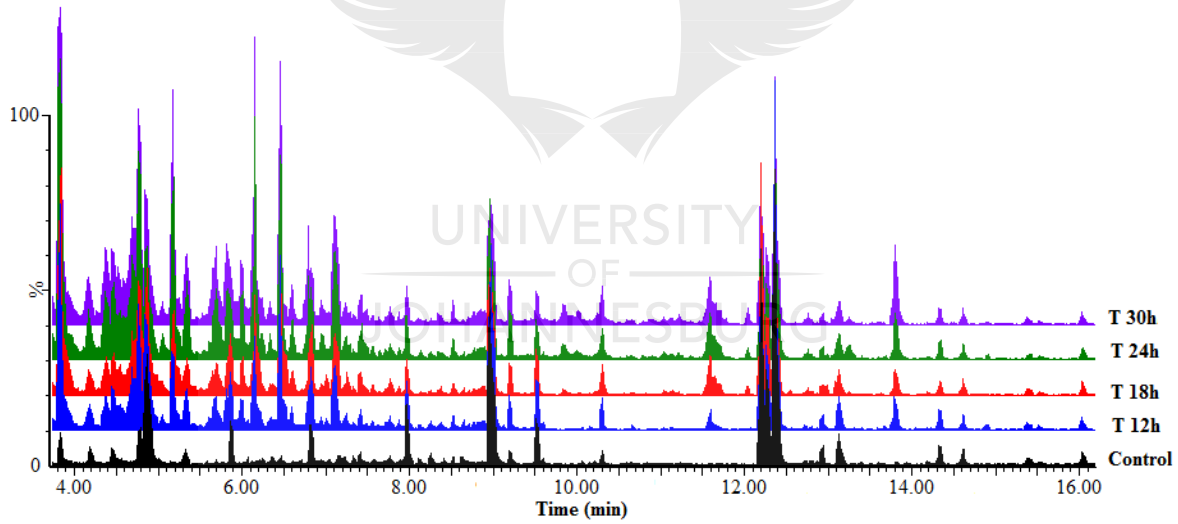

Figure S 5.2: UHPLC-MS BPI chromatograms (ESI positive) of methanolic <u>extracellular</u> extracts of sorghum cells treated with LPS*B. andr.* The chromatograms of a control (non-treated 0 h) *vs* treated samples (0-30 h) display variation related to treatment- and time-related metabolic changes occurring in the cells due to LPS treatment.

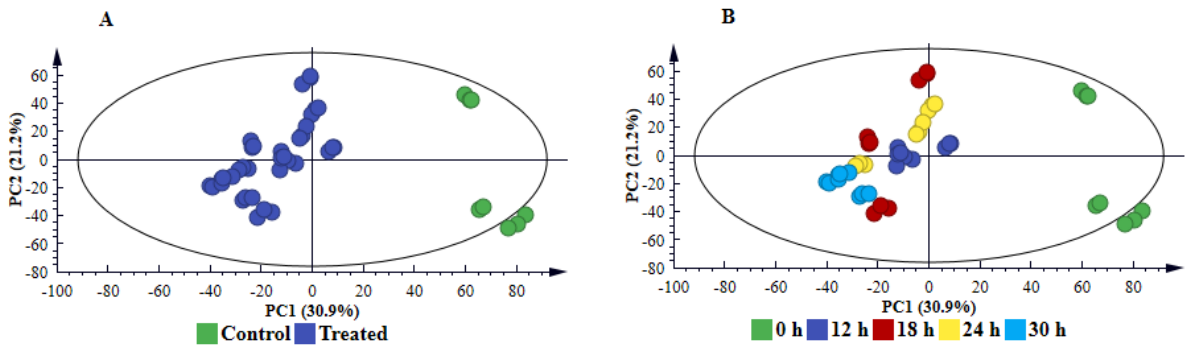

Figure S 5.3: PC analyses of the LC-MS (ESI positive data) for intracellular sorghum cell extracts. The 5-component model, explains 68.6% variations in Pareto-scaled data, X, and the amount of predicted variation by the model, according to cross-validation, is 57.3%. The first 2 PCs were used to generate the above scores plot of all data. **A:** Clusters coloured based on condition *i.e.* non-treated/treated shows clear separation between treated and non-treated samples. **B** is the same scores plot but coloured according to time

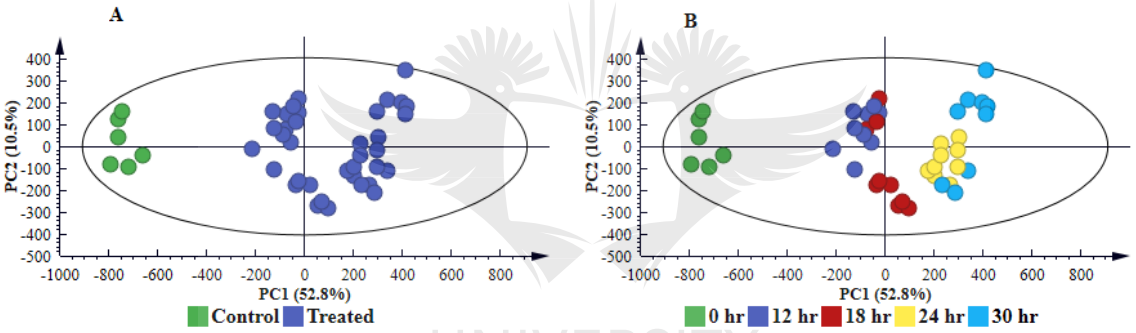

Figure S 5.4: PC analyses of the LC-MS (ESI positive data) for extracellular sorghum cell extracts. The 4-component model, explains 78.6% variations in Pareto-scaled data, X, and the amount of predicted variation by the model, according to cross-validation, is 54.2%. The first 2 PCs were used to generate the above scores plot of all data. **A:** Clusters coloured based on condition *i.e.* non-treated/treated shows clear separation between treated and non-treated samples. **B** is the same scores plot but coloured according to time.

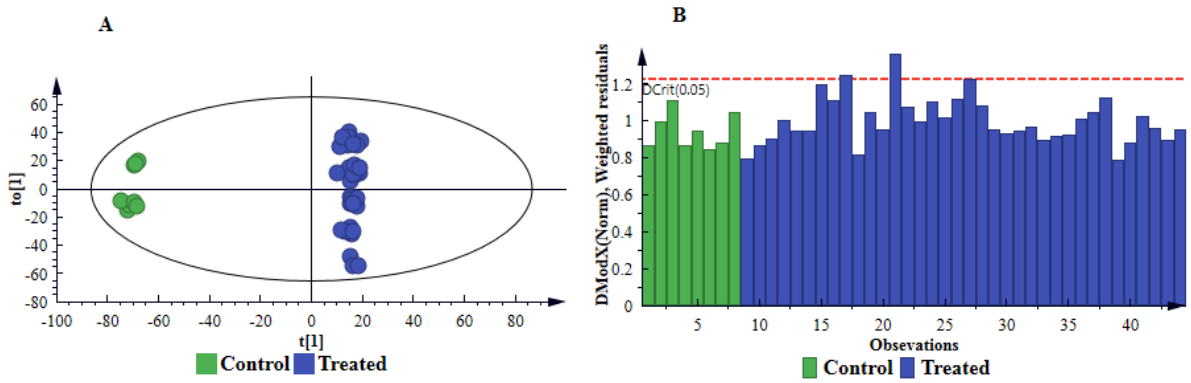

Figure S 5.5: Supervised multivariate analyses of the LC-MS (ESI positive data) for <u>intracellular</u> extracts. (A): Grouping of control (C0 h) *vs* treated (all time points combined) as indicated by an OPLS-DA score plot. This model comprises 1 predictive component and 3 orthogonal components (R^2X= 58.2%, R^2Y= 99.6% and Q^2= 95.7%). **(B):** A distance to the model in space X (DModX) plot to detect outliers (above the dashed red line, Dcrit) in the OPLS-DA scores plot

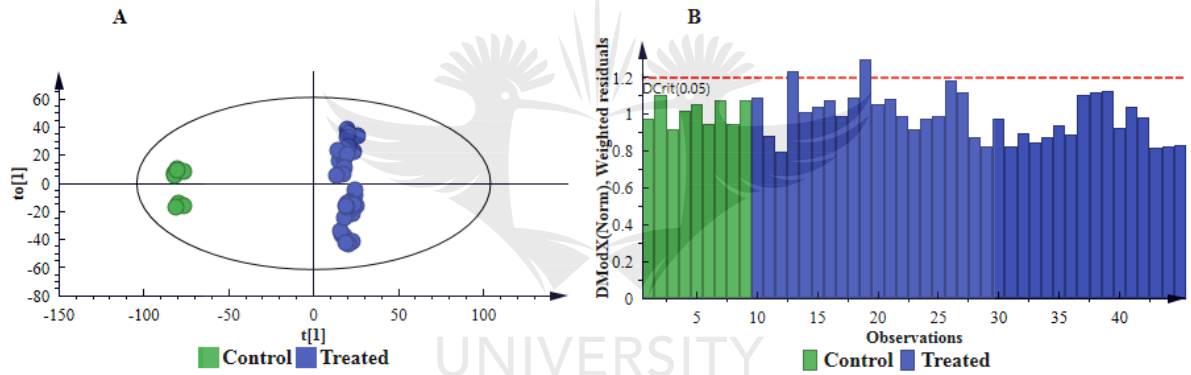

Figure S 5.6: Supervised multivariate analyses of the LC-MS (ESI positive data) for <u>extracellular</u> extracts (excluding QCs). (A): Grouping of control (C0h) *vs* treated (all time points combined) as indicated by an OPLS-DA score plot. This model comprises 1 predictive component and 2 orthogonal components (R^2X= 47.1%, R^2Y= 99.5% and Q^2= 94.0%). **(B):** A distance to the model in space X (DModX) to detect outliers (above the dashed red line, Dcrit) in the OPLS-DA scores plot.

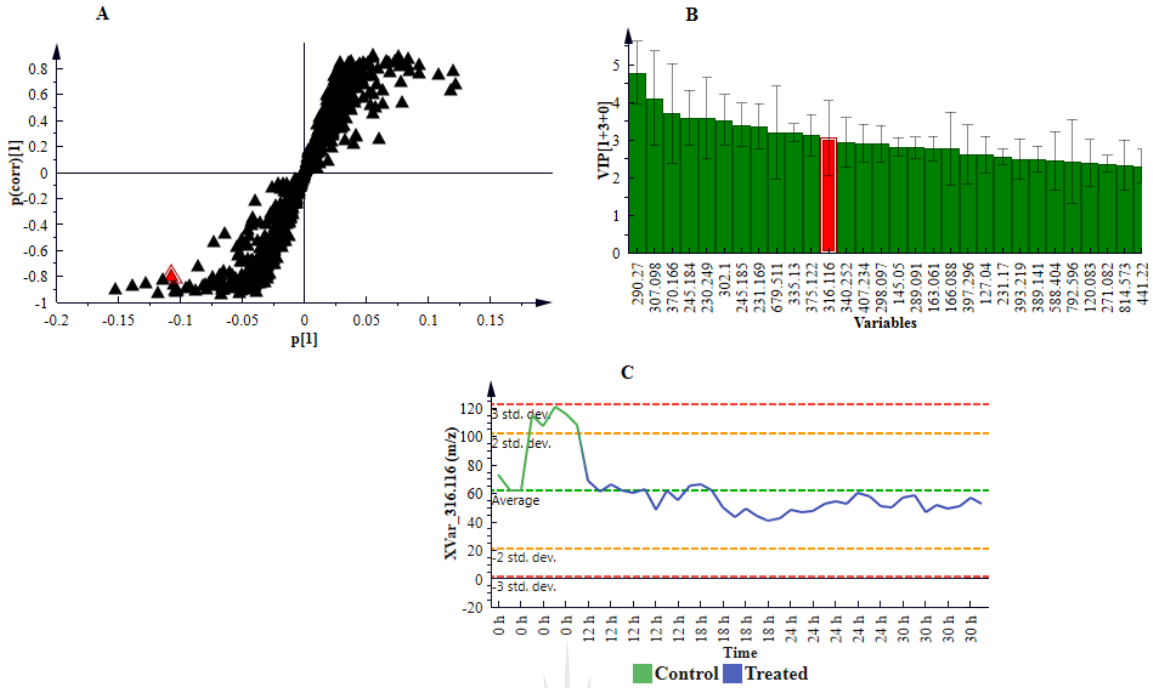

Figure S 5.7: OPLS-DA modelling analyses of the LC-MS (ESI positive data) for <u>intracellular</u> extracts (excluding QCs). (A) An OPLS-DA loading S-plot displaying the discriminating features (ions) that explain the clustering (sample grouping) observed in the OPLS-DA scores plot with the features in the top right quadrant positively correlated to the treatment and those in the bottom left quadrant negatively correlated to the treatment. **(B)** A VIP plot summarising the importance of some of the variables in the projection of the model with the *m/z* values and standard deviations indicated. A VIP value >1 is significant/important in the projection and increase in value indicates an increase in significance of the variable. **(C)** Representative variable trend plot displaying the changes of the selected variable across the samples.

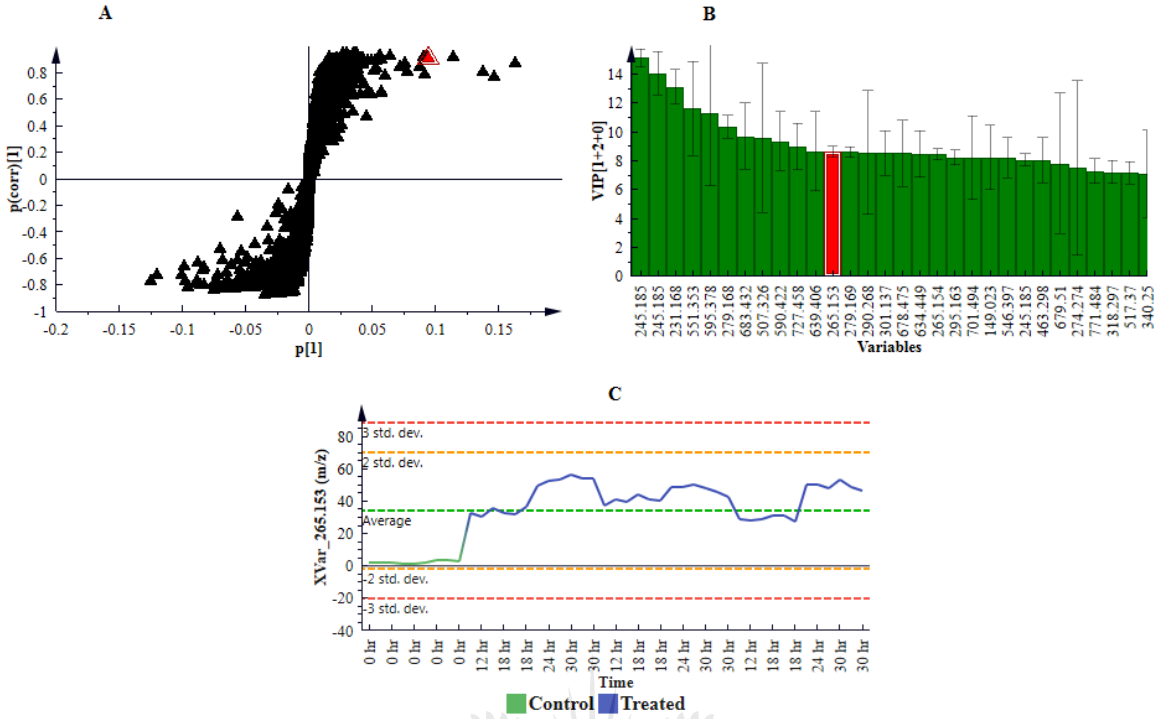

Figure S 5.8: OPLS-DA modelling analyses of the LC-MS (ESI positive data) for <u>extracellular</u> extracts (excluding QCs). (A) An OPLS-DA loading S-plot displaying the discriminating features (ions) that explain the clustering (sample grouping) observed in the OPLS-DA scores plot with the features in the top right quadrant positively correlated to the treatment and those in the bottom left quadrant negatively correlated to the treatment. **(B)** A VIP plot summarising the importance of some of the variables in the projection of the model with the *m/z* values and standard deviations indicated. A VIP value >1 is significant/important in the projection and increase in value indicates an increase in significance of the variable. **(C)** Representative variable trend plot displaying the changes of the selected variable across the samples.

Table S 5.1: Annotated discriminatory metabolites from <u>intracellular extracts</u> of LPS*B. andr.*-treated *Sorghum bicolor* cultured cells, displaying the fold changes at different time points. The summarised metabolites were annotated at MI-level 2 and had VIP scores > 1. Fold changes were obtained from an OPLS-DA model computed of control 0 h *vs* treated 12h, 24 h and 30 h. (*Data for the 18 h time point is presented in the main text*).

Metabolites	*m/z*	Rt (min)	Adduct	Ion mode	Formula	C0 h vs T12 h		C0 h vs T24 h		C0 h vs T30 h	
						p-value	Fold change	*p*-value	Fold change	*p*-value	Fold change
Sorgoleone	359.2298	4.98	$[M+H]^+$	pos	$C_{22}H_{30}O_4$	0.017	2.9	0.002	3.3	0.006	4.3
L-phenylalanine	164.0686	1.84	$[M-H]^-$	neg	$C_9H_{11}NO_2$	0.015	1.2	1.28E-06	1.6	0.000	1.4
L-Tryptophan	203.0798	2.78	$[M-H]^-$	neg	$C_{11}H_{12}N_2O_2$	3.89E-06	1.3	7.12E-07	1.5	7.37E-05	1.4
15-Hydroxylinoleic acid	295.2253	14.29	$[M-H]^-$	neg	$C_{18}H_{32}O_3$	8.38E-05	2.3	0.533	1.2	0.119	1.4
Dihydroxyoctadecadienoic acid	311.2242	11.79	$[M-H]^-$	neg	$C_{18}H_{32}O_4$	0.001	7.4	1.54E-05	5.4	2.75E-10	7.1
9,10-Dihydroxy-12-octadecenoic acid	313.2354	12.67	$[M-H]^-$	neg	$C_{18}H_{34}O_4$	1.24E-11	17.9	0.386	2.0	7.36E-05	5.3
9,10-Dihydroxystearic acid	315.2511	13.51	$[M-H]^-$	neg	$C_{18}H_{36}O_4$	1.12E-06	3.9	4.20E-05	4.2	2.48E-09	4.5
Trihydroxyoctadecadienoic acid I	327.2149	9.72	$[M-H]^-$	neg	$C_{18}H_{32}O_5$	0.439777	5.2	0.072	29.4	0.381	10.5
Trihydroxyoctadecadienoic acid II	327.2135	11.05	$[M-H]^-$	neg	$C_{18}H_{32}O_5$	1.67E-06	197.9	3.16E-12	49.3	4.44E-06	138.8
9,12,13-Trihydroxy-10-octadecenoic acid	329.2327	9.60	$[M-H]^-$	neg	$C_{18}H_{34}O_5$	0.001	1.6	0.089	1.3	0.044	1.3
16-Hydroxypalmitate	273.2553	13.65	$[M+H]^+$	pos	$C_{16}H_{32}O_3$	0.037	1.7	0.702	0.9	0.253	0.7
Sophoraflavanone G	423.1821	4.42	$[M-H]^-$	neg	$C_{25}H_{28}O_6$	0.001	0.9	0.000	0.8	2.76E-05	0.8
Apigenin-8-C-glucoside (vitexin)	431.0974	5.58	$[M-H]^-$	neg	$C_{21}H_{20}O_{10}$	0.202	0.7	0.176	0.7	0.140	0.7
Apigenin-6-C-xyloside-8-C-glucoside (vicenin-1)	565.1545	4.94	$[M+H]^+$	pos	$C_{26}H_{28}O_{14}$	0.112	0.7	0.025	0.5	0.036	0.6
Apigenin-6,8-di-C-glucoside (vicenin-2)	595.1687	4.77	$[M+H]^+$	pos	$C_{27}H_{30}O_{15}$	0.101	0.4	0.030	0.2	0.022	0.2
Apigenin 7,4'-dimethyl ether	316.1157	8.29	$[M+H\_NH_3]^+$	pos	$C_{17}H_{14}O_5$	0.001	0.6	0.000	0.5	0.000	0.6
3',4'5-Trihydroxy-3,7-dimethoxyflavone	367.0221	3.90	$[M-H]^-$	neg	$C_{17}H_{20}O_9$	0.068	0.6	0.003	0.6	0.067	0.8
4-Coumaroyl-3-hydroxyagmatine	291.1471	5.72	$[M-H]^-$	neg	$C_{14}H_{20}N_4O_3$	0.008	0.5	0.001	0.6	0.767	1.0
4-Coumaroylquinic acid	337.1474	1.77	$[M-H]^-$	neg	$C_{16}H_{18}O_8$	7.02E-05	0.8	3.73E-07	0.7	3.62E-09	0.6
Cinnamoylserotonin	351.1251	2.43	$[M-H\_HCOOH]^-$	neg	$C_{19}H_{18}N_2O_2$	0.000	0.9	5.97E-07	0.7	1.74E-06	0.8
Feruloylserotonin	351.1266	2.86	$[M-H]^-$	neg	$C_{20}H_{20}N_2O_4$	4.60E-06	0.8	0.985	1.0	0.527	1.6
Sinapaldehyde glucoside	369.1199	3.61	$[M-H]^-$	neg	$C_{17}H_{22}O_9$	0.781	1.1	0.040	0.5	0.069	0.8
1-O-Coumaroyl-beta-D-glucose	371.0957	4.94	$[M-H\_NaNa]^-$	neg	$C_{15}H_{18}O_8$	1.97E-07	0.8	1.25E-12	0.5	1.21E-12	0.5
Sinapyl alcohol	209.0764	6.72	$[M-H]^-$	neg	$C_{11}H_{14}O_4$	1.31E-06	3.5	6.93E-07	5.3	2.87E-05	3.6
Dihydroconiferyl alcohol glucoside	413.1422	3.27	$[M+H\_HCOONa]^+$	pos	$C_{16}H_{24}O_8$	0.006	0.7	0.001	0.5	0.006	0.7

Indole-3-butyric acid	272.0893	2.84	$[M+H\_HCOONa]^+$	pos	$C_{12}H_{13}NO_2$	0.000	0.5	8.13E-06	0.6	5.71E-07	0.5
N(6)-[(Indol-3-yl)acetyl]-L-lysine	304.1667	4.20	$[M+H]^+$	pos	$C_{16}H_{21}N_3O_3$	1.11E-07	5.2	1.42E-06	4.7	1.14E-09	6.7
Indole-3-acetyl-myo-inositol	353.1348	2.44	$[M-H\_NH_3]^-$	neg	$C_{16}H_{19}NO_7$	0.427	0.9	0.624572	0.9	0.114	0.8
Indole-3-acetyl-beta-1-D-glucoside	382.1121	3.93	$[M-H\_HCOOH]^-$	neg	$C_{16}H_{19}NO_7$	0.074	0.7	0.009	0.4	0.064	0.7
6-Hydroxy-indole-3-acetyl-valine	291.1294	3.89	$[M+H]^+$	pos	$C_{15}H_{17}N_2O_4$	0.056	0.3	0.023	0.2	0.136	0.5
Traumatic acid	297.1291	3.90	$[M+H\_HCOONa]^+$	pos	$C_{12}H_{20}O_4$	0.000	7.4	0.524	0.5	0.189	3.1
(9R,13R)-1a,1b-Dihomo-jasmonic acid	239.1638	12.19	$[M+H]^+$	pos	$C_{14}H_{22}O_3$	0.034	0.8	0.750	1.0	0.001	0.7
Zeatin-7-beta-D-glucoside	397.1826	6.73	$[M-H\_NH_3]^-$	neg	$C_{16}H_{23}N_5O_6$	0.079	0.7	0.018	0.7	0.084	0.8
Zeatin	220.1197	2.15	$[M+H]^+$	pos	$C_{10}H_{13}N_5O$	6.94E-05	0.6	4.39E-06	0.5	7.74E-06	0.5
Methyl jasmonate	247.1298	2.52	$[M+H\_Na]^+$	pos	$C_{13}H_{20}O_3$	1.31E-08	3.4	5.94E-11	3.9	1.31E-11	5.2
Dihydrozeatin riboside	354.1769	6.20	$[M+H]^+$	pos	$C_{15}H_{23}N_5O_5$	0.001	0.4	0.001	0.4	0.006	0.5
Zeatin riboside	374.1463	5.39	$[M+H\_Na]^+$	pos	$C_{15}H_{21}N_5O_5$	0.001	0.4	0.000	0.2	4.24E-05	0.1
Azelaic acid	187.0935	6.74	$[M-H]^-$	neg	$C_9H_{16}O_4$	2.45E-17	4.1	1.64E-17	5.1	2.29E-12	4.6
Abscisic acid	265.1552	3.35	$[M+H]^+$	pos	$C_{15}H_{20}O_4$	2.55E-08	3.5	1.18E-09	2.8	2.34E-09	4.0
Agmatine	173.0787	5.52	$[M-H\_NaNa]^-$	neg	$C_5H_{14}N_4$	1.74E-06	1.3	0.000	1.2	0.001	1.7
Riboflavin	377.1476	4.49	$[M+H]^+$	pos	$C_{17}H_{20}N_4O_6$	0.241135	0.8	0.000	0.3	0.001	0.4

Table S 5.2: Annotated discriminatory metabolites from extracellular extracts of LPS*B. andr.*-treated *Sorghum bicolor* cultured cells, displaying the fold changes at different time points. The summarised metabolites were annotated at MI-level 2 and had VIP scores > 1. Fold changes were obtained from an OPLS-DA model computed of control 0 h *vs* treated 12h, 24 h and 30 h. (*Data for the 18 h time point is presented in the main text*).

Metabolites	*m/z*	Rt (min)	Adduct	Ion mode	Formula	C0 h vs T12 h		C0 h vs T24 h		C0 h vs T30 h	
						p-value	Fold change	*p*-value	Fold change	*p*-value	Fold change
Sorgoleone	359.2298	4.98	$[M+H]^+$	pos	$C_{22}H_{30}O_4$	0.006	18.3	3.01E-09	45.6	3.09E-06	43.4
L-phenylalanine	164.0686	1.84	$[M-H]^-$	neg	$C_9H_{11}NO_2$	3.07E-11	2.7	1.04E-12	2.6	5.87E-12	2.6
L-Tryptophan	203.0798	2.78	$[M-H]^-$	neg	$C_{11}H_{12}N_2O_2$	0.001	1.4	0.000	1.4	2.14E-05	1.5
Dihydroxyoctadecadienoic acid	311.2242	11.79	$[M-H]^-$	neg	$C_{18}H_{32}O_4$	0.110	5.2	0.002	4.9	0.012	4.2
Trihydroxyoctadecadienoic acid II	327.2135	11.05	$[M-H]^-$	neg	$C_{18}H_{32}O_5$	0.001	22.7	0.158	52.4	4.02E-11	53.4
9,12,13-Trihydroxy-10-octadecenoic acid	329.2327	9.60	$[M-H]^-$	neg	$C_{18}H_{34}O_5$	1.13E-08	9.2	2.82E-09	8.4	1.13E-10	6.8
Sophoraflavanone G	423.1821	4.42	$[M-H]^-$	neg	$C_{25}H_{28}O_6$	0.030	1.1	0.054	1.1	0.028	1.1
Apigenin-8-*C*-glucoside (vitexin)	431.0974	5.58	$[M-H]^-$	neg	$C_{21}H_{20}O_{10}$	0.129	1.3	0.057	1.3	0.079	1.3
Apigenin-6-C-xyloside-8-C-glucoside (vicenin-1)	565.1545	4.94	$[M+H]^+$	pos	$C_{26}H_{28}O_{14}$	0.006	1.3	0.002	1.4	0.002	1.4
Apigenin-6,8-di-C-glucoside (vicenin-2)	595.1687	4.77	$[M+H]^+$	pos	$C_{27}H_{30}O_{15}$	0.873	1.3	0.159	2.0	0.014	2.70
4-Coumaroyl-3-hydroxyagmatine	291.1471	5.72	$[M-H]^-$	neg	$C_{14}H_{20}N_4O_3$	8.17E-05	2.8	4.32E-06	3.4	4.25E-07	4.0
4-Coumaroylquinic acid	337.1474	1.77	$[M-H]^-$	neg	$C_{16}H_{18}O_8$	0.138475	1.1	0.129	1.1	0.013	1.1
Cinnamoylserotonin	351.1251	2.43	$[M-H\_HCOOH]^-$	neg	$C_{19}H_{18}N_2O_2$	2.81E-05	1.2	1.28E-06	1.3	7.71E-06	1.2
Feruloylserotonin	351.1266	2.86	$[M-H]^-$	neg	$C_{20}H_{20}N_2O_4$	0.738	1.3	0.520	1.6	0.185	3.1
Sinapaldehyde glucoside	369.1199	3.61	$[M-H]^-$	neg	$C_{17}H_{22}O_9$	1.54E-06	2.7	2.61E-08	3.3	3.73E-09	3.6
1-O-Coumaroyl-beta-D-glucose	371.0957	4.94	$[M-H\_NaNa]^-$	neg	$C_{15}H_{18}O_8$	0.001	1.8	6.03E-05	1.9	8.81E-07	2.3
Sinapyl alcohol	209.0764	6.72	$[M-H]^-$	neg	$C_{11}H_{14}O_4$	5.80E-12	3.5	1.64E-10	2.8	1.73E-06	2.2
Indole-3-acetyl-myo-inositol	353.1348	2.44	$[M-H\_NH_3]^-$	neg	$C_{16}H_{19}NO_7$	0.086	2.2	0.007	2.6	0.001	2.6
Indole-3-acetyl-beta-1-D-glucoside	382.1121	3.93	$[M-H\_HCOOH]^-$	neg	$C_{16}H_{19}NO_7$	0.941	0.9	0.494	0.9	0.407	0.9
6-Hydroxy-indole-3-acetyl-valine	291.1294	3.89	$[M+H]^+$	pos	$C_{15}H_{17}N_2O_4$	0.000	2.7	3.67E-05	3.0	1.23E-05	3.3
Azelaic acid	187.0935	6.74	$[M-H]^-$	neg	$C_9H_{16}O_4$	2.04E-17	5.5	4.47E-15	4.1	6.58E-09	3.6
Abscisic acid	265.1552	3.35	$[M+H]^+$	pos	$C_{15}H_{20}O_4$	1.26E-13	16.2	1.24E-15	23.7	3.71E-15	22.9
Riboflavin	377.1476	4.49	$[M+H]^+$	pos	$C_{17}H_{20}N_4O_6$	2.54E-06	4.2	5.27E-08	7.1	2.07E-10	7.4

Chapter 6: Concluding Remarks

One of the growing interests and key research focus in plant science is to sustainably develop stress-tolerant/resistant crops as an ultimate goal in improving crop production. The latter is beneficial in sustaining the rapidly expanding population as well as boosting the world economy. One sustainable crop protection strategy is to genetically increase tolerance/resistance through the exploitation of natural secondary metabolite defences of plants as highlighted in **Chapter 1**. However, this necessitates identification of the associated metabolic signatures that form part of the plant chemical defence arsenal. In plant studies, the untargeted metabolomics approach has emerged as an indispensable tool in identification and analysis of these molecular signatures, particularly since the metabolome best mirrors the physiological state of a plant under a specific physiological condition (**Chapter 2**). Thus, the work in this study provided vital information regarding defence-related secondary metabolites in *S. bicolor*, of which production can be manipulated in order to increase sorghum pathogen resistance.

Chapter 4, highlighted on the metabolic reprogramming in two *S. bicolor* cultivars, NS 5511 and NS 5655, induced by *B. andropogonis* infection. Following monitoring of the metabolic changes over time (1-9 d.p.i.), metabolomic analyses revealed that the 'defensomes' of the two cvs spanned an array of defence-related metabolites arising from different metabolic pathways, aimed to establish an enhanced defensive state. Here, *S. bicolor* metabolites belonging to primary metabolism (amino acids and fatty acids) and secondary metabolism (benzoates, cyanogenic glycoside, flavonoid, hydroxycinnamic acids, indoles, carboxylic acids and shikimates) were annotated. Alterations in a range of phytohormones of the salicylates, jasmonates and zeatins classes were noted, which correlate to observed differential changes in the metabolite pools. The results obtained herein reveal the significant diversity and extensive adaptive capabilities of *S. bicolor* in stress responses. Qualitatively, the dominant presence of flavonoids and hydroxycinnamic acids confirms their significant role in sorghum defence. Furthermore, the study demonstrated that the two cvs employ similar defence mechanisms at a metabolic level, as evidenced by the presence of the same metabolite classes. However, the varying concentrations (relative quantities) and time of accumulation of crucial defence-related metabolites (such as salicylates and apigenin - and derivatives) significantly contributed to the

state of resistance. Herein, as also revealed by the disease severity-rating index, the NS 5511 was found to be more resistant compared to the NS 5655 cv since crucial defence metabolites displayed an earlier accumulation as well as elevated levels. This study provides an insight into the metabolic reprogramming in *S. bicolor* in response to *B. andropogonis* infection, and highlights the metabolic signatures involved in the plant's defence to this particular bacterial pathogen. Thus, the work significantly contributes to expanding the current knowledge regarding bacterial sorghum–pathogen interactions (as the knowledge is limited), and in uncovering molecular signatures that define biochemical processes involved in *S. bicolor* responses to bacterial infection by *B. andropogonis.*

In a live plant–pathogen interaction, the host plant would respond to a mixture of different MAMPs as pathogen-derived elicitors. In order to investigate the effect of a specific MAMP on metabolomic reprogramming in sorghum, a reductionist approach was followed where sorghum cells in culture were treated with lipopolysaccharides (LPS) purified from *B. andropogonis.* The compositional and structural analysis of the LPS revealed that the MAMP with M_r > 7.5 kDa comprised of all three structural components, *i.e.* the O-antigen, core oligosaccharide and Lipid A (**Chapter 5**). The isolation and purification of this *B. andropogonis*-derived elicitor, represents the first for the LPS from this particular species. Metabolomic analyses of the cultured cells system revealed the metabolic reprogramming in *S. bicolor* suspensions triggered by *B. andropogonis* LPS (**Chapter 5**) to be similar to that in plant leaf tissue, triggered by the live pathogen (*i.e.* similar metabolite classes, that include amino acids, flavonoids, hydroxycinnamic acids, fatty acids, indoles and phytohormones - **Chapter 4**). However, there were some differences in the quality and quantity of metabolites comprising the metabolomes. Additionally, the cultured cell suspension system revealed secretion of metabolites into the medium following LPS elicitation. This might be correlated to translocation of defence metabolites to the cell periphery/apoplast for defence-related functions as would occur upon pathogen challenge of plants.

As briefly outlined above, a comparison between the metabolic reprogramming in plant leaf tissue following live *B. andropogonis* treatment (**Chapter 4**) and that of sorghum cells in culture following LPS elicitation (**Chapter 5**), revealed that cells responded to the *B. andropogonis*-derived MAMP in a qualitatively similar manner as *B. andropogoni*s- treated plant leaf tissue. Although much overlap was observed between the quality of the metabolomes and the metabolic pathways altered following *B. andropogonis* and LPS treatment in plants

and cell cultures, respectively, some variances were observed. The identification of the allelochemical, sorgoleone, and other metabolites in cultured cells (undifferentiated) but absent in the plant leaf tissue systems (differentiated), demonstrated the phenomenon of specialised cells/tissue specific synthesis of some secondary metabolites and/or associated with a certain stage of development. These differences demonstrate the chemo-diversity of the two biological systems, influenced by factors such as the state of tissue differentiation and developmental stage. Additionally, the differing quantity of annotated secondary metabolites between the two systems can be attributed to differentiated tissue synthesising more secondary metabolites than undifferentiated cells. Even though there were some differences between two systems, the results depicted in the cell culture system confirm the *B. andropogonis*-derived LPS as one of the important instrumental factors eliciting immune responses in *S. bicolor*.

Finally, UHPLC-HDMS-based untargeted metabolomics proved to be a useful and powerful platform for the comprehensive identification and analysis of an array of chemically diverse metabolites, spanning a wide range of metabolic pathways associated with sorghum defence responses.