
Metabolomic Analysis of Oat (*Avena sativa*) Plants: A Strategy for Cultivar Identification and for the Characterisation of Host Responses to Pathogen Infection

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With God all things are
possible.

-Matthew 19:26

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Preface

Preface

Aspects of the research from this MSc study, related to the content in this dissertation, have been presented in the following:

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List of Abbreviations

ABA	Absciscic acid
Avns	Avenanthramides
BPI	Base peak intensity
CE	Capillary electrophoresis
CV-ANOVA	Cross-validation analysis of variation
d.p.i.	Days post-inoculation
ESI	Electron spray ionisation
ETI	Effector-triggered immunity
FT-ICR	Fourier-transform ion cyclotron resonance
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
HR	Hypersensitive response
ISR	Induced systemic resistance
JA	Jasmonic acid
LC	Liquid chromatography
MAS	Marker-assisted selection
M/PAMP	Microbe/pathogen-associated molecular patterns
MS	Mass spectrometry
<i>m/z</i>	Mass-to-charge ratio
NMR	Nuclear magnetic resonance
OPLS-DA	Orthogonal projection to latent structures-discriminant analysis
PBS	Phosphate buffered saline
PCA	Principal component analysis
PLS-DA	Partial least squares-discriminant analysis
PTI	Pathogen-triggered immunity
pv.	Pathovar
QC	Quality control

QTOF	Quadrupole time-of-flight
ROC	Receiver operator characteristic
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
T3SS	Type III secretion system
UHPLC	Ultra-high performance liquid chromatography
VIP	Variable importance in projection
1D	One-dimensional
2D	Two-dimensional
3D	Three-dimensional

Units

Da	Dalton
eV	Electron volt
g	Gram
mg	Milligram
µg	Microgram
mL	Milliliter
µL	Microliter
mg/L	Milligram per liter
mM	Millimolar
µM	Micromolar
°C	Degrees celsius
h	Hour
min	Minute
Rt	Retention time
%	Percentage

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Summary

Oat as a cereal crop has a range of health benefits associated with its consumption, most of which are attributed to the vast range of phytochemicals that it contains such as phenolic acids, flavonoids, phytosterols, carotenoids, avenanthramides, avenacosides and avenacins. Not only do these compounds offer a range of human health benefits, but also play an important role in the growth, development and survival of the plant when exposed to environmental or pathogenic threats. The metabolome, being the final recipient of genetic information and biological information flow, gives a glimpse into the cellular environment of the plant as reflected in the type and concentrations of molecules, and their interactions. The study of the metabolome thus allows for the detection and assessment of a broad range of metabolites and has great value in both phenotyping and diagnostic analyses in plants. The application of metabolomics has thus found its way into plant breeding, biotechnology and to improve resistance/ tolerance mechanisms in plants.

In this study, metabolomics was applied to unravel differential metabolic profiles of various oat (*Avena sativa* L.) cultivars and to identify signatory biomarkers for cultivar identification and for oat response to bacterial infection. To achieve this, two separate studies were carried out. Firstly, the respective cultivars (Magnifico, Dunnart, Pallinup, Overberg, and SWK001) were grown to seedling stage where the leaves and roots were harvested for cultivar metabolic profiling studies. Secondly, metabolic alterations involved in oat responses to inoculation with the bacterial pathogen, *Pseudomonas syringae* pv. *coronafaciens*, were investigated by infecting two of the five cultivars (Dunnart and SWK001, differing in disease susceptibility) at seedling stage and harvesting the leaves from 1 to 4 days post-inoculation (d.p.i.). Metabolites were extracted for both the cultivar profiling and bacterial inoculation studies using an 80% methanol extraction method. All methanolic extracts were analysed using an ultra-high-performance liquid chromatography (UHPLC) system coupled to a quadrupole-time-of-flight (qTOF) high definition mass spectrometer analytical platform. The generated data were processed and analysed using multivariate statistical methods (PCA and OPLS-DA) to extract interpretable information.

Based on the results, the cultivars were successfully distinguished from one another (in metabolic profiles derived from both leaves and roots) and signatory biomarkers were identified for cultivar profiling which included carboxylic acids, amino acids and fatty acids. Secondary metabolites included a range of phenolic compounds (hydroxycinnamic- and hydroxybenzoic acids and associated derivatives) as well as flavonoids. In response to bacterial infection, signatory biomarkers were identified that included amino acids, fatty acids / lipids, alkaloids, terpenoids, saponins, phenolics (phenolic acid amides and flavonoids) and plant hormones. These identified metabolic markers not only revealed signatory metabolic profiles that allowed for the differentiation among the respective oat cultivars but also revealed metabolic reprogramming that lead to dynamic alterations of the metabolic profiles associated with plant defence.

Chapter 1: General Introduction

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1.1 Problem statement

Food demand has been rapidly increasing with the overall growth in the world population. Now more than ever, crop improvement and plant breeding studies have become imperative in ensuring food security and sustainability. Therefore, new crop varieties that can produce greater yields, and withstand biotic and abiotic stresses, are imperative. Developing new crop varieties can, however, be an extremely lengthy process. Biotechnology has significantly reduced the time it takes to create new crop varieties where various tools have been developed to simplify and reduce the trait selection process. Such tools include DNA-based methods, referred to as marker-assisted selection (MAS). Although MAS has shown success in crop improvement, it is also faced with many limitations like the fact that the presence of a gene does not ensure the expression of the trait. Metabolomics has the potential to overcome this limitation and provide useful insights about metabolites involved in growth, development, resistance and stress responses which, in turn, can be applied for crop improvement.

1.2 Background

Oat belongs to the monocotyledonous *Poaceae* family along with other cereals like wheat, rice, barley, rye, maize, sorghum and millet (Sarwar *et al.*, 2013). Among these cereals, oat has gained increasing popularity due to numerous health and nutritional benefits involved in both human and livestock consumption (Jing & Hu, 2012; Rasane *et al.*, 2015). Some of these benefits include the reduction of serum cholesterol levels, plasma insulin response, glucose

uptake, incidences of certain types of cancers and cardiovascular diseases (Singh *et al.*, 2013; Bao *et al.*, 2014; Boffetta *et al.*, 2014; Hou *et al.*, 2015; Grundy *et al.*, 2018). Oat has also gained popularity in agriculture due to its hardiness and ability to thrive and withstand environmentally poor conditions where other cereals seem to be lacking (Saini *et al.*, 2019). Many of oat benefits are due to the numerous bioactive phytochemicals (secondary metabolites) therein such as phenolic acids, flavonoids, phytosterols and carotenoids, as well as oat specific compounds – avenanthramides, avenacosides and avenacins (Belobrajdic & Bird, 2013; Sang & Chu, 2017).

Over the years, analytical technologies have been greatly improved to comprehensively and unbiasedly study the underlying metabolic profiles of a plant. These profiling technologies have led to the simultaneous detection of hundreds of metabolites in a single plant extract, thus piecing together the metabolome (Liu & Locasale, 2017). Metabolomics thus provides a means of generating a ‘chemical fingerprint’ of the biological and biochemical mechanisms involved in plant growth, development and the mechanisms impacted by biotic or abiotic factors (Tan *et al.*, 2016). Due to the sessile nature of plants, they depend on a range of primary and secondary metabolites for growth, development and survival (Wang *et al.*, 2019). Generally, the former is directly involved in plant growth, and the latter in response to biotic and abiotic stresses as well as adaptation to environmental changes (Isah, 2019). To deal with such stresses plants have developed an advanced immune system that includes innate immunity and inducible responses such as systemic acquired resistance (SAR), induced systemic resistance (ISR) and priming (Tugizimana *et al.*, 2018).

When plants are exposed to pathogens, two possible outcomes can occur namely compatible- or incompatible interactions. Compatible interactions ensue when the pathogen successfully infects the plant, conversely, the opposite is true for incompatible interactions where the plant successfully wards off the pathogen and avoids infection (Gupta *et al.*, 2015; Ponzio *et al.*, 2016). The final outcome of plant-pathogen interactions therefore depends on the balance between the ability of the pathogen to suppress the plant’s immune response, and the ability of the plant to recognise and counter the pathogen attack by activating effective defences (Balint-Kurti, 2019). A more holistic understanding of plant defence mechanisms, that metabolomics has the potential to provide, could lead to the development of plant breeding strategies to increase disease resistance in plants (Kumar *et al.*, 2017).

Plant breeding is mostly driven by the need to improve plant characteristics through the creation of desired genotypes and phenotypes in new cultivars (Bhargava & Srivastava, 2019). Oat plant breeding programs have been aimed at maximising yields, however, many different factors may influence crop yields such as pests, diseases and environmental factors (Bailey-Serres *et al.*, 2019). Susceptibility to these influences can affect both the yield and quality, and therefore finding ‘ideal’ characteristics in crop varieties are essential (Tester & Langridge, 2010). In this regard, the identification of cultivars and their tolerance and susceptibility traits become an extremely important aspect in agricultural systems (Korir *et al.*, 2013). The identification of cultivars and varieties is not only essential for finding favourable traits, but are also extremely important steps during plant breeding, registration, trade, inspection and seed production. A rapid and effective fingerprinting method is therefore required for early cultivar identification, where metabolomics seems to offer great potential in this regard (Xu & Crouch, 2008; Korir *et al.*, 2013).

Ultimately, the study of secondary metabolites through metabolomics has the potential to provide new insights into the underlying biochemistry of the plant, which can be used as a valuable tool in plant breeding for early cultivar identification and for the evaluation of defence-related traits. In this study, metabolomics was used to profile oat cultivars as a means of developing an early cultivar identification method. As such, metabolomics was applied to identify signatory biomarkers that can be used for oat cultivar identification and for the identification of defence-related traits by analysing methanolic extracts on an UHPLC-qTOF-MS system.

1.3 Aims and objectives

Aims:

- Distinguish between various oat cultivars through the identification and evaluation of discriminatory markers from their metabolic profiles / metabolomes.
- Metabolically profile oat response to bacterial infection so as to identify and characterise secondary metabolite markers related to the oat defence response to the halo blight pathogen.

Objectives:

- ‘Metabo-phenotyping’ of oat cultivars (Dunnart, Overberg, Pallinup, Magnifico and SWK001) that differ in a range of agronomic properties.
- Metabolomic analysis and metabolite profiling of oat cultivars (leaves and roots) using untargeted, high-resolution liquid chromatography – mass spectrometry as an analytical platform.
- Comparison of the profiles with regard to the presence or absence of separated compounds based on chromatographic properties, retention times, peak intensities and molecular masses.
- Identification of signatory biomarkers for cultivar identification and differentiation using multivariate statistical tools and chemometric methods.
- Elucidation of metabolite-based host defences based on alterations in oat after bacterial inoculation with *Pseudomonas syringae* pv. *coronafaciens* (Ps-c).
- Identification of potential metabolic biomarkers for the defence-related response of oat cultivars (Dunnart and SWK001) to pathogen inoculation.

1.4 Dissertation outline

Chapter 2: Literature review

Chapter 3: Plant metabolomics for biomarker discovery: Key signatory metabolic profiles for the identification and discrimination of oat cultivars.

Chapter 4: Plant metabolomics for the characterization of halo blight disease in oat cultivars caused by *Pseudomonas syringae* pv. *coronafaciens*.

Chapter 5: General conclusion and future prospects.

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Chapter 2: Literature Review

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Oat (*Avena sativa* L.)

2.1 Oat (*Avena sativa* L.): Importance as a grain crop

In the next 50 years, the world population will increase exponentially, meaning more crops will have to be produced, however, in less optimal conditions such as water shortage and adverse environmental conditions in order to ensure food security (Berners-Lee *et al.*, 2018). To meet these challenges, yield potential of food crops needs to be improved while combating abiotic and biotic stresses caused by climate change. It is therefore imperative that environmental factors affecting plant growth are understood and managed to prevent loss, damage and increase yields (Pandey *et al.*, 2017). Cereals, cultivated for their edible grains, are rich in essential nutrients, energy and vitamins. Due to the rich nutritional value, cereals are considered the core of the human diet (McKevith, 2004; Sarwar *et al.*, 2013; Papageorgiou & Skendi, 2018). Oat belongs to the monocotyledonous *Poaceae* family along with other cereals like wheat, rice, barley, rye, maize, sorghum and millet (Sarwar *et al.*, 2013). Not only are these cereals produced in greater quantities than any other type of crop(s), they also provide the greatest amount of food energy worldwide (Awika, 2011). Of these cereals, rice, maize and wheat are the most popular, conversely, oat has only recently gained popularity due to numerous health and nutritional benefits involved in both human and livestock consumption (Jing & Hu, 2012; Rasane *et al.*, 2015). It is also considered a superior cereal crop due to its hardiness and ability to thrive and withstand environmentally poor conditions where other cereals seem to be lacking (Saini *et al.*, 2019). Due to these benefits, oat has attracted the attention of scientific research where numerous studies found that, in addition to the high nutritional value, increased consumption also reduces serum cholesterol levels, plasma insulin response and glucose uptake. Moreover, oat consumption has been reported to reduce incidences of certain types of cancers and cardiovascular diseases (Singh *et al.*, 2013; Bao *et al.*, 2014; Boffetta *et al.*, 2014; Hou *et al.*, 2015; Grundy *et al.*, 2018). Many of these benefits are due to the numerous bioactive phytochemicals (secondary metabolites) that oat contains such as phenolic acids, flavonoids, phytosterols and carotenoids, to name a few. Additionally, this cereal also produces unique phytochemicals known as avenanthramides, avenacosides and avenacins (Belobrajdic & Bird, 2013; Sang & Chu, 2017).

2.1.1 Oat development

Oat as a crop goes through distinct developmental stages from planting to harvesting. To describe these stages, the Zadoks scale is commonly used, although other scales have been developed such as the Feekes, BBCH (*Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie*), Haun, Kuperman and Semenova, and Keller-Baglioni scale (Tejera & Heaton, 2017). The Zadoks growth scale is an internationally used system applied for research and agricultural processes. The scale has ten primary growth stages that can also be divided into ten secondary stages, and hence a scale of 00-99. The primary growth phases include germling, seedling, tillering, jointing, booting, flowering and mature grain (**Figure 2.1**). These phases are hallmarked by certain characteristics which include the appearance of the coleoptile in the germling stage (code 0), the appearance of the first, second and third leaf in the seedling phase (code 1), and the tillering phase (code 2) which involves the formation of new stems that are independent of the main shoot. Next, the jointing and stem elongation (code 3) is associated with the appearance of the first node and extends into the development of the flag leaf, followed by booting (code 4 and 5) and is characterised by the emergence of the ear from the boot and ends off in a fully emerged flag leaf. Stem swelling then occurs in preparation for the flowering phase. Flowering (code 6) is associated with the formation of anthers for grain development which is subsequently divided into milk development (code 7), dough development (code 8) and finally the ripening (code 9) of the caryopsis into a mature seed or grain (Zadoks *et al.*, 1974; Thomas, 2014; Barber *et al.*, 2015). These types of classification systems have great importance in agriculture and are often used as a guideline for monitoring growth stages before making any cropping decisions such as when to apply pesticides.

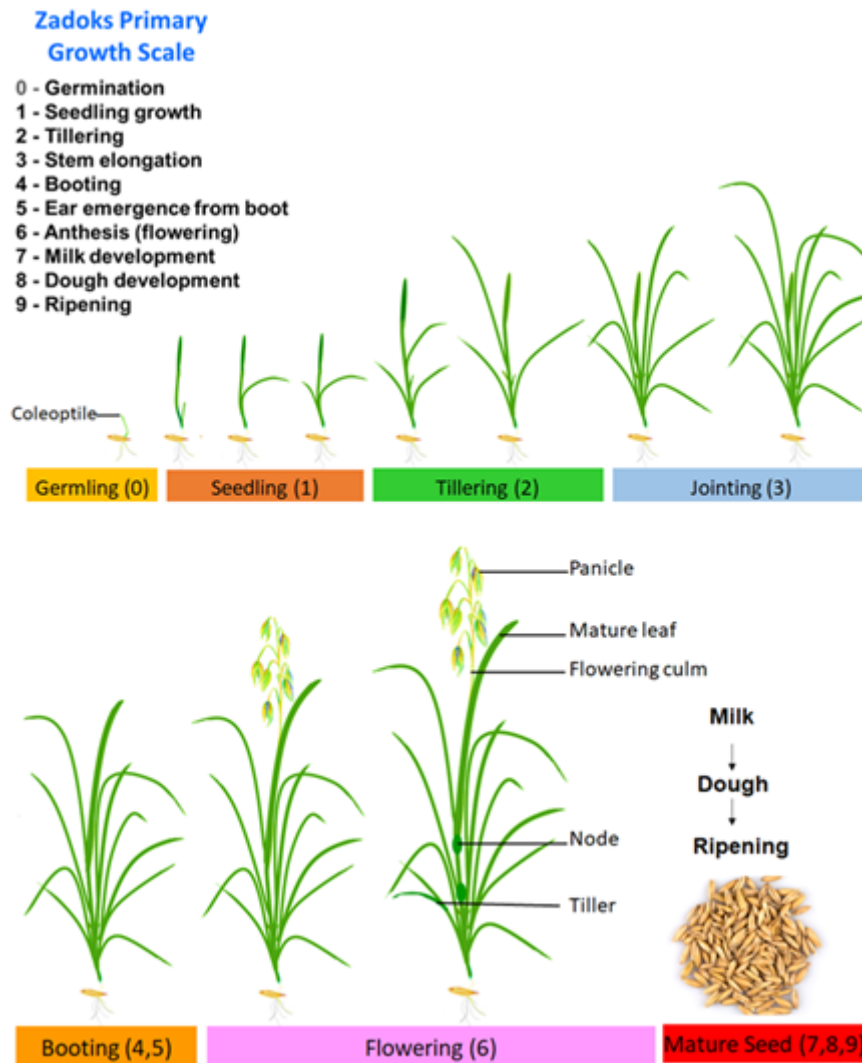


Figure 2.1: Recognised growth stages of oat. The Zadoks decimal growth scale illustrates the ten primary growth stages of oat crops from germination to maturity.

Environmental factors can affect plants at a molecular, developmental, morphological, and physiological level, and it is therefore imperative to understand the complex interactions that occur between the plant and environment in order to predict the phenotype (Pandey *et al.*, 2017). To elucidate the interaction of the environment on crops, a new concept of “envirotyping” has been proposed, which is complementary to genotyping and phenotyping. The purpose of envirotyping is to contribute to crop modelling, phenotype prediction, genotype-by-environment interaction and to study the effect of biotic and abiotic stresses on crops. Envirotyping, therefore, has a range of applications, such as environmental characterisation, genotype-by-environment analysis, phenotype prediction, plant breeding and to elucidate the relationship between the genotype, phenotype, environment, and the developmental phase (Xu, 2016). Since changes in the plant metabolome are at the core of

plant development and ultimately reflect the phenotype, studying the metabolome could play an essential role in underpinning how a plant responds to the environment and in determining phenotypic plasticity (Brunetti *et al.*, 2013; Sharma *et al.*, 2018). Phenotypic plasticity can be described as a plant's ability to exhibit multiple phenotypes governed by a single genotype when present in different environments (Gratani, 2014). To measure phenotypic plasticity, the development (morphological traits) and function (metabolic traits) of the plant have to be monitored. Since metabolomics has already proven to be key in elucidating metabolic fingerprints during different plant growth and developmental phases, it has endless possibilities in predicting the plant phenotype and its interaction with the environment (Fusco & Minelli, 2010). To study these complex interactions, plant materials are ideally extracted at an early developmental stage once the correlation between metabolite levels and the trait of interest becomes apparent. This not only reduces screening costs but also allows for easy harvesting, handling, and analysis (Fernandez *et al.*, 2016).

2.1.2 Oat production and uses

South Africa is one of the smaller producers of oat with the majority produced, processed and used locally. Production rates average from 39 thousand tons per annum depending on various factors such as environmental conditions, plantings and prices of competing crops. South Africa, therefore, remains a net importer of oat, importing an average of 22 thousand tons per year to meet local demand. As a winter crop oat is optimally suited for winter rainfall areas such as the Western Cape which is responsible for approximately 96% of South African oat production, followed by the Free State (3%) and the Northern Cape (1%) (South African Department of Agriculture, Forestry and Fisheries, 2016; Sihlobo, 2018). Oat is grown for grain, forage, bedding, pasture or as a rotational crop depending on the regions in the world. In South Africa, oat is primarily used for livestock feed and a variety of human consumables (Stevens *et al.*, 2004). Among the range of cereals, oat is distinguished for their resistant traits and often grown in crop rotation to break cycles of soil-borne pathogens (Soriano *et al.*, 2004). Resistant oat crops are also less vulnerable to pathogen and disease infections and can additionally be used to minimise rusts, smuts and blights. Oat has also gained popularity as a cover crop due to its ability to meet numerous soil conservation priorities (Lu *et al.*, 2000). Cover crops function in protecting soil from erosion, preventing nutrient loss and improving crop production and productivity. For a crop to be classified as a cover crop it should be able

to germinate quickly, tolerate diverse environmental conditions, absorbs soil nutrients, tolerates pests and diseases, and most importantly not outcompete the main crop (Fageria *et al.*, 2005; Reddy, 2016). Oat as a cover crop produces natural allelopathic chemical compounds in their roots that suppress weed growth; additionally, they take up excess soil nutrients, prevent erosion through their deep fibrous root system, and can work in conjunction with other cover crops (Cheng & Cheng., 2015). Knowledge of characteristics is important when choosing crops for planting. Agricultural advancements have allowed the identification of crop characteristics such as their uses, yield and tolerance to different pathogens and diseases. **Table 2.1** shows the characteristics and resistance, or susceptibility status of the various oat cultivars used throughout this study.

Table 2.1: Characteristics, common uses and resistance or susceptibility status of five oat cultivars (ARC, 2020; Agricol, 2020).

Cultivars	Grain yield	Type	Lodging resistance *	Plant height (cm)	Crown rust resistance	Stem rust resistance	Main use
Overberg	Good	Winter	Good	80	S	MS	Grain/ Grazing
Pallinup	High	Spring	Good	80	MS	MS	Grain
Magnifico	Good	Winter	Average	100+	Unk	Unk	Grazing/ Forage
Dunnart	Good	Winter/ Spring	Good	80	MR	S	Grazing
SWK 001	Average	Winter/ Spring	Average	100+	MR	MS	Grazing

*R = Resistant; MR = Moderately resistant; MS = Moderately susceptible; S = Susceptible; Unk = Unknown. Puccinia coronata (crown rust) and Puccinia graminis (stem rust) cause rust diseases in oat. * Lodging in grain crops can be described as the bending of the stem causing difficulties in harvesting and results in yield reduction.*

2.1.3 Oat phytochemicals and health benefits

Over the past few years, interest in oat for human consumption has gained increasing popularity due to the wide variety of bioactive phytochemicals, such as avenanthramides, avenacosides, β -glucans, tocopherols, sterols, and phytic acids, to name a few. These compounds have been found to contribute to the reduction of cholesterol, cardiovascular diseases, diabetes, gastrointestinal disorders and certain types of cancers (Gangopadhyay *et al.*, 2015; Martinez-Villaluenga & Peñas, 2017). Avenanthramides (AVA) are a group of hydroxycinnamoyl anthranilate alkaloids, uniquely found in oat and known to have antioxidant, anti-inflammatory and anti-proliferative activities in humans (Lee-Manion *et al.*, 2009). Many different avenanthramides have been identified with AVA-A (2p), -B (2f) and -C (2c) being most abundantly produced (de Bruijn *et al.*, 2019). Oat is also the only saponin-containing cereal and produces unique steroidal glycosides known as avenacoside A and B. These compounds have been found to exhibit anticancer activity by inhibiting tumour growth through cell cycle arrest and apoptosis (discussed in more detail in section 2.3.6) (Moses *et al.*, 2014; Martinez-Villaluenga & Peñas, 2017). Oat β -glucans have positive effects on human health such as improving blood cholesterol levels and reducing diabetes. These cereal β -glucans have a particular structure that generates viscosity in the intestinal tract, which is thought to be the reason for their positive health effects (Henrion *et al.*, 2019). Various antioxidant compounds have been associated with oat health benefits, such as tocopherols, (*i.e.* tocopherols and tocotrienols), sterols and phenolics (Peterson, 2001). Tocopherols function as strong free radical scavengers and can restrict the proliferation of certain cancer cells (Shahidi & De Camargo, 2016). Oat also contains other antioxidant phytochemicals namely sterols and phytic acids that exhibit antioxidant activity by chelating metal ions and making it catalytically inactive (Martinez-Villaluenga & Peñas, 2017). Cereals like oat also have two major types of phenolic acids that function as antioxidants, namely hydroxycinnamic acids or hydroxybenzoic acids. Hydroxycinnamic acids include ferulic -, coumaric -, caffeic - and sinapic acids while hydroxybenzoic acids include protocatechuic -, syringic -, vanillic -, and gallic acids (Soycan *et al.*, 2019).

2.1.4 *Pseudomonas syringae* pv. *coronafaciens*

Pseudomonas syringae is one of the most common Gram-negative phytopathogenic bacteria. *P. syringae* was first isolated from infected plants, with over 60 pathovars (pvs.) currently identified, and each pathovar represents a different strain infecting a specific host. Collectively, these pathovars infect most economically important plants and could potentially threaten global crop productions (Lamichhane *et al.*, 2014; Xin *et al.*, 2018; Gutiérrez-Barranquero *et al.*, 2019). They have therefore gained increasing interest in scientific research and have been extensively studied as a model for understanding bacterial pathogenicity and molecular mechanisms of plant-pathogen interactions (Starkey & Rahme, 2009; Sitaraman, 2015). As a pathogen, *P. syringae* populates and causes disease in the intracellular spaces of leaves in a wide variety of crops. To successfully suppress plant innate immunity and cause disease, these pathogens inject effector proteins into the host cells through a type III secretion system (T3SS). Recent studies have shown that pathogen-triggered immunity (PTI) defence responses restrict the injection of type III effectors into the host cell to counteract pathogen spread (Oh *et al.*, 2010; Xin *et al.*, 2018). The outcome of infection thus depends on the interaction between the pathogen by effector deposition into the plant and the subsequent plant response with R proteins (as discussed in **section 2.5.2.2**). The T3SS mechanisms are responsible for the injection of effectors and are not involved in the actual activation or suppression of plant defence (Anderson *et al.*, 2014). Some genes encoding effector proteins have been identified in the pathovar responsible for causing halo blight disease in oat crops (*P. syringae* pv. *coronafaciens*); such as *avrPto*, *avrD1*, *avrAE1*, *hopA1*, *hopB1*, *hopD1*, and *hopAF1* genes. These effector genes are important for producing pathogen effectors and their presence generally result in successful infection (Dutta *et al.*, 2018). Previous studies have reported on the response of oat to *P. syringae* pv. *coronafaciens* as resulting in hypersensitive response. The HR was characterised by the breakdown of inoculation sites (necrosis) from 24 to 36 h post-inoculation, followed by drying around the site of infection 3 to 4 days after inoculation. After 10 days post-infection the symptoms can be described as the characteristic lesions surrounded by yellow halos (Smith & Mansfield, 1981) (**Figure 2.2**).



Figure 2.2: Halo blight disease in oat crops. Typical developed symptoms of oat leaves infected by *Pseudomonas syringae* pv. *coronafaciens* (taken from Malik *et al.*, 2011; Byamukama, 2019).

The pathovar known as *P. syringae* pv. *coronafaciens* or *P. coronafaciens* has been identified to cause halo blight disease in oat crops worldwide (Dutta *et al.*, 2018). It was first reported back in 1920 (Elliot, 1920) and was later reported in Wales, Sweden, Norway, New Zealand, Kenya and Canada, where damage to crops and yield losses were severely observed in the colder, humid regions (Griffiths & Peregrine, 1956; Wilkie, 1972; Harder & Harris, 1973; Martens *et al.*, 1985; Persson & Sletten, 2001). The most recent report of halo blight in oat occurred in South Korea, where the characteristic symptoms were apparent (Kim, 2020). These symptoms are typically characterised by small, oval-shaped, water-soaked spots found on leaves in early onset of the disease, and soon change to a reddish-brown, oval-shaped lesion with a light centre and a characteristic yellow halo surrounding the lesion (**Figure 2.2**). Rising temperatures have been known to produce more pronounced halos, whereas cooler temperatures are associated with a more characteristic brownish lesion (Persson & Sletten, 2001). As a phytopathogenic bacterium, *P. syringae* is seedborne and can survive on infected crop residues and subsequently spread from leaf to leaf and plant to plant through rain, wind and insects, particularly aphids (Martinelli, 2000; Lamichhane *et al.*, 2014). Over the years, it has become apparent that this pathogen has the potential to impair crop health and result in great economic losses under favourable conditions, which is worrisome especially with global climates constantly changing.

Metabolomics

2.2 Plant Metabolomics

It is well known that plants produce a great number of chemical compounds, and are probably the most effective and exquisite organisms on earth. One plant can produce roughly twenty-five thousand compounds at any given time and, additionally, it is speculated that more than one million different metabolites may exist in the plant kingdom (Weckwerth & Fiehn, 2002; Beckles & Roessner, 2012; Wang *et al.*, 2019). These chemical compounds are imperative for the sustainability and survival of plants under various environmental conditions and are just as important for our survival as they directly (food, fuel, and pharmaceuticals) and indirectly (livestock feed and fodder) sustain our life on earth. It is therefore justified to say that our existence on earth depends on plants and their metabolic capacity (Beckles & Roessner, 2012).

Recent advances in analytical technologies have made it possible to comprehensively and unbiasedly study the underlying biochemical inventory of a plant. These profiling technologies have led to the simultaneous detection of hundreds of metabolites in a single plant extract, thus putting the pieces of the puzzle together to elucidate the metabolome (Liu & Locasale, 2017). From a systems biology viewpoint, the metabolome is found downstream of the genome, transcriptome and proteome (Idle & Gonzalez, 2007), and offers a functional readout of the cellular state of a biological system (Rinschen *et al.*, 2019). Metabolomics thus provides a means of generating a ‘chemical fingerprint’ of endogenous biological processes impacted by environmental factors or stresses (Tan *et al.*, 2016). Although the metabolome embodies an enormous group of chemical compounds (that exhibits wide differences in structure and function), they are classified into two groups namely primary or secondary metabolites (Hussein & El-Anssary, 2018). Primary metabolites constitute the greatest portion of the metabolome and are essential compounds immediately required and readily available for plant survival under normal growth conditions. Secondary metabolites, on the other hand, are produced to facilitate interaction with the environment and to enhance the plant’s survival under unfavourable conditions involving biotic - and abiotic stresses (Isah, 2019; Wang *et al.*, 2019). Since metabolites directly reflect biochemical processes, their qualitative and quantitative analysis allows new insight into the biochemical systems involved in producing

the phenotype, whilst also yielding a comprehensive metabolic profile of the particular system (Ramirez *et al.*, 2013; Tugizimana *et al.*, 2013; Hong *et al.*, 2016). Metabolomics thus provides a non-biased characterisation of the plant metabolome and can reveal the correlation between genes and the biochemical composition of plant tissue in response to the environment (phenotype) and can consequently be used to assess gene function (genotype) (**Figure 2.3**) (Fiehn, 2002; Hamany Djande *et al.*, 2020).

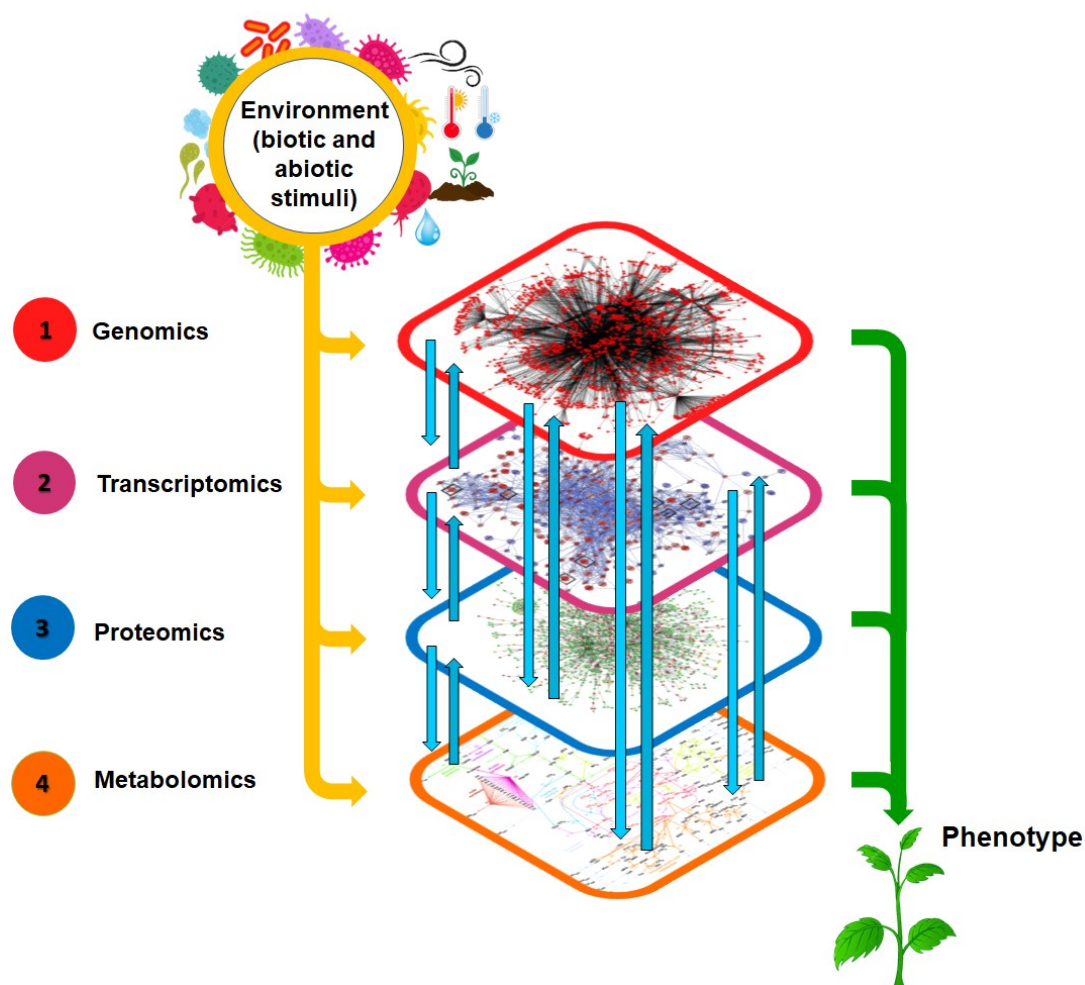


Figure 2.3: Systems biology schematic showing the biological flow of information and the technological platforms from the genome to the phenome. The metabolome and its metabolite constituents are considered the underlying biochemical layer showing all information expressed and modulated throughout the other omics layers, making it the closest link to the phenotype (Hamany Djande *et al.*, 2020).

Due to the diverse and complex makeup of the metabolome, a range of analytical methodologies have been developed to comprehensively study the metabolome. Metabolite profiling, one such an approach, has been developed for the identification and quantification of large groups of metabolites. In the context of metabolomic analysis, fingerprinting can be described as a rapid non-invasive high-throughput analysis method that provides phenotypic

characterisation and distinction between specific metabolic states (Liang *et al.*, 2015). Accordingly, metabolomic fingerprinting, has gained increasing popularity for analysis of the metabolome (Kopka *et al.*, 2004; Ellis *et al.*, 2007; Llanes *et al.*, 2018). For a more in-depth view of the extracellular and intracellular metabolites, produced by organisms, metabolic footprinting has been popular (Ellis *et al.*, 2007; Tugizimana *et al.*, 2013). Metabolomic strategies can also be divided into untargeted, targeted and semi-targeted approaches. Untargeted metabolomics can be described as the comprehensive analysis of all analytes and chemical unknowns in a single sample and produces a multitude of metabolic profiles that can be applied to quantify relative concentrations under different conditions or across populations. Conversely, targeted metabolomics is described as the measurement of annotated metabolites and chemically characterised groups in a sample with a well-defined or specific hypothesis in mind (Ellis *et al.*, 2007; Tugizimana *et al.*, 2013; Cambiaghi *et al.*, 2017; Ribbenstedt *et al.*, 2018; Llanes *et al.*, 2018). Lastly, semi-targeted can be described as an approach to quantitatively and tentatively identify predefined metabolites without a predefined hypothesis (Liu *et al.*, 2017).

2.2.1 Metabolomics workflows

The first step in a plant metabolomics study, after constructing the biological question, is the experimental or study design. The experimental procedure should therefore be planned thoroughly to ensure that the derived analytical data answer the proposed biological question (Jacyna *et al.*, 2019; Rodrigues *et al.*, 2019). The study design, therefore, encompasses planning all experimental details such as plant growth conditions, extraction protocol, sample analysis, data analysis, and statistical analysis. The experimental procedure for a basic metabolomics study can be categorised into three main steps namely sample preparation, data acquisition and data analysis (Verpoorte *et al.*, 2008; Wang *et al.*, 2010; Tugizimana *et al.*, 2013).

2.2.1.1 Sample preparation

Sample preparation is a crucial step in a metabolomics study and initially starts with harvesting the plant material, quenching metabolic activity, extracting, and preparing the sample for analysis. During this process, care must be taken to avoid contamination or degradation of the

sample as this could affect the accuracy, outcome and biological interpretation down the line (Kim & Verpoorte, 2010; Tugizimana *et al.*, 2013). The process of harvesting plant material is often rapid due to the rate of metabolite turnover in living systems (Riekeberg & Powers, 2017). Therefore, immediately after harvesting, the plant material is quenched (inactivation of biochemical and enzymatic processes) by either snap freezing with liquid nitrogen, adding hot or cold methanol or changing the pH with perchloric acid, to name a few (Biais *et al.*, 2011; Jorge *et al.*, 2016). The quenching method is usually chosen based on the type of sample and how the sample will be analysed. Once the material has been harvested and quenched the extraction process follows. As discussed, the plant metabolome is known to be highly complex, and full coverage is consequently restricted by sensitivity and selectivity of the analytical technique as well as sample preparation methods (Wang *et al.*, 2010). When extracting metabolites, the extraction solvent chosen should thus be as comprehensive as possible to answer the biological question whilst still maintaining the chemical stability of the metabolites (Jorge *et al.*, 2016). The inherent selectivity of the solvent is usually considered when choosing an appropriate extraction solvent or combination of solvents. In untargeted plant metabolomics, a non-selective solvent is often sought after in an attempt to acquire the greatest number of metabolites possible. Solvents such as methanol and acetonitrile are commonly used as they can extract polar (hydrophilic) as well as non-polar (hydrophobic) compounds (Martin *et al.*, 2014; Liu & Locasale, 2017). Extraction methodologies also often include liquid extraction (temperature- or pressure-assisted), solid-phase extraction (SPE) and microwave-assisted extraction (MAE) (Zhang *et al.*, 2012; Altemimi *et al.*, 2017). Environmentally safe extractants such as natural deep eutectic solvents (NADES), pressurised hot water extraction (PHWE) and aqueous two-phase extraction solvents (ATPs) are also available (Khoza *et al.*, 2014; Owczarek *et al.*, 2016; Hamany Djande *et al.*, 2018). For a comprehensive view of the metabolome, different extraction methodologies are often combined due to the lack of a defined extraction protocol allowing coverage of the entire metabolome (Zhang *et al.*, 2012; Mushtaq *et al.*, 2014; Tugizimana *et al.*, 2014).

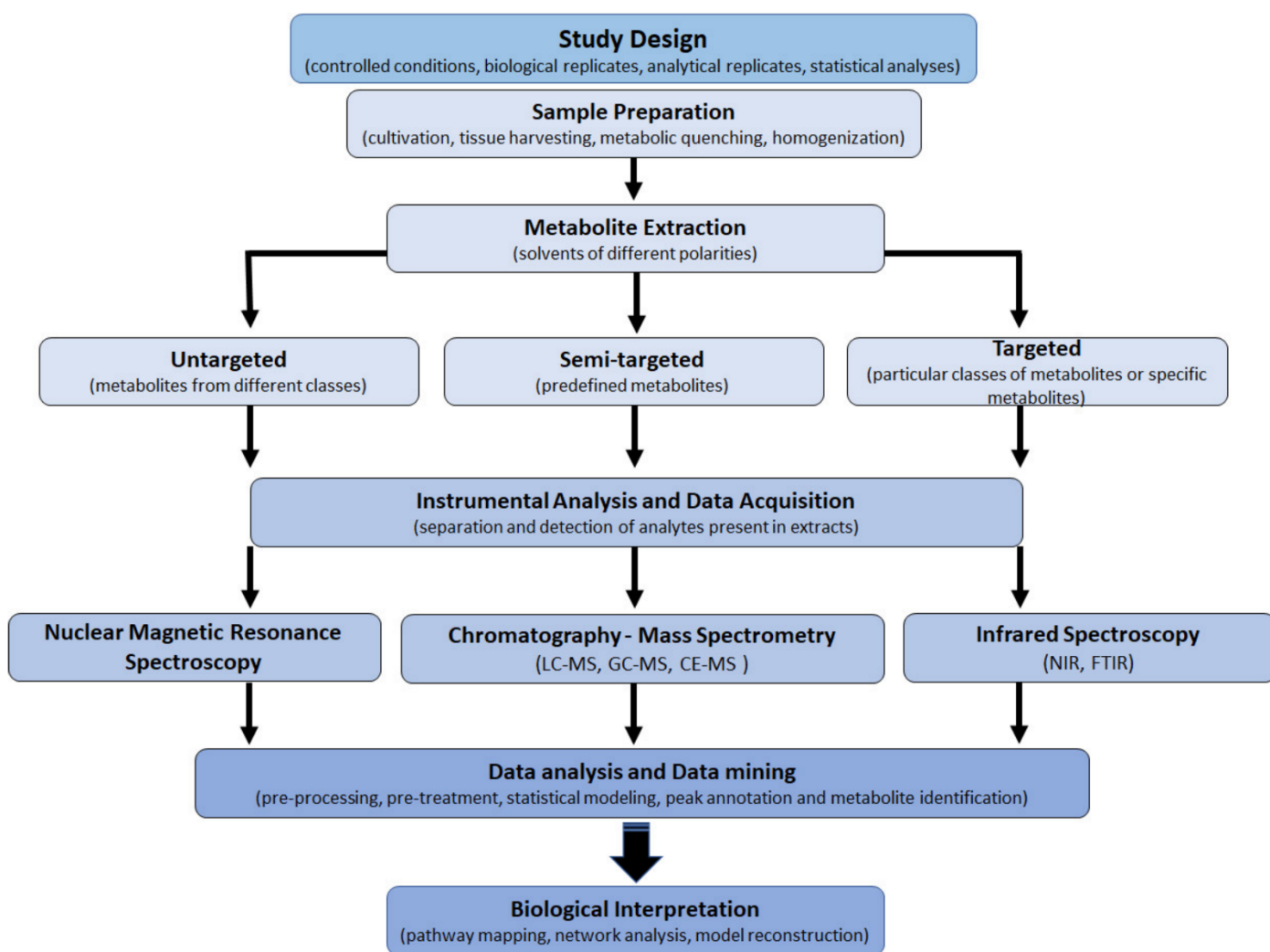


Figure 2.4: Typical multi-step plant metabolomics workflow: from experimental design and sample preparation to biological interpretation. Sample preparation, data acquisition and data analysis are known to form the core of metabolomics analysis. These steps are all interrelated and subsequently come together to produce biological information in the form of networks and pathways for a comprehensive view of the metabolome (Hamany Djande *et al.*, 2020).

2.2.1.2 Data acquisition

After sample preparation, a variety of bioanalytical platforms are assessed for data acquisition and are often based on mass spectrometry (MS) or nuclear magnetic resonance (NMR) techniques (Emwas *et al.*, 2019). Fourier transform infrared spectroscopy (FTIR) has also gained a reputation in the metabolomics field, thanks to its ability to swiftly and simply analyse and characterise complex building blocks simultaneously (Gilany *et al.*, 2014; Junot & Fenaille, 2019). The output of metabolomics largely depends on the methodologies and instrumentation used; therefore, the appropriate analytical platform is essential for obtaining

reliable and reproducible data (Dunn & Ellis, 2005). Various analytical platforms have therefore been developed and are often coupled for a more thorough analysis such as GC-MS (gas chromatography coupled to mass spectrometry detection), LC-MS (liquid chromatography coupled to mass spectrometry detection), CE-MS (capillary electrophoresis- mass spectrometry) and FT-ICR-MS (Fourier-transform ion cyclotron resonance mass spectrometry) (Okazaki & Saito, 2012). Analytical platforms are chosen based on the class(es) of metabolites to be analysed as well as the sensitivity and selectivity of the different platforms (Wang *et al.*, 2010). NMR spectroscopy has offered many advantages for metabolomics analysis such as rapid, highly reproducible methods with little sample preparation and high throughput. Unfortunately, it is also faced with many limitations such as low sensitivity and resolution that subsequently affects the number of metabolites identified and annotated (Nagana Gowda & Raftery, 2017; Emwas *et al.*, 2019).

MS, comparatively, has been coupled to numerous platforms and provides a rapid, sensitive and selective qualitative and quantitative analysis (Jorge *et al.*, 2016; Wishart, 2019). LC x LC / GC x GC (2D) and multidimensional LC / GC have gained popularity as analytical techniques by combining multiple columns having distinct stationary phase selectivity to provide greater resolution and higher peak capabilities (Stoll *et al.*, 2018). These newly established systems allow for real-time analysis of the metabolome and lipidome and are highly efficient for large-scale metabolomics studies where sample amounts are limited. MS-based platforms have therefore been invaluable in the detection and identification of metabolites by providing spectral data such as fragmentation patterns and possible corresponding molecular formulae. However, unambiguous identification of the molecular structures and identities of some unknown compounds remains a bottleneck in metabolomics research. The development of complex hyphenated techniques such as LC-MS-NMR; 2D-LC-MS; 2D-GC-MS, 2D-GC-Q-Orbitrap-MS, among others, offers improved spectral resolution and metabolite identification capabilities (Kapoor *et al.*, 2016; Walker *et al.*, 2016). In this study, an ultra-high-performance liquid chromatography system coupled to a high definition quadrupole time-of-flight mass spectrometer (UHPLC-QTOF-MS) was used for analysis. This hyphenated system offers a strong alternative to conventional HPLC-MS/MS in terms of its high resolution, low costs, reduced run time and high resolving power (Lamuela-Raventós *et al.*, 2014; Chawla & Ranjan, 2016). It also increases sensitivity by using column (stationary phase) material made up of extremely small particle sizes ($< 2 \mu\text{m}$) and ultimately increases throughput whilst

offering real-time analysis (Nguyen *et al.*, 2006; Taleuzzaman *et al.*, 2015). UHPLC-MS has therefore been extremely popular as the platform of choice for metabolomics studies.

2.2.1.3 Data mining and chemometrics

Metabolomics studies often yield large-scale, complex data sets that require advanced data analytical tools for efficient analysis. Statistics, cheminformatics and bioinformatics are essential for a comprehensive assessment of these datasets (Cambiaghi *et al.*, 2017). Another crucial step in metabolomics workflows, data mining, involves data pre-processing and pre-treatment, chemometrics, multivariate statistical analyses, and metabolite annotation. Data pre-processing methods include noise filtering, peak detection, retention time (Rt) correction, and peak alignment (Aksenov *et al.*, 2017). Additionally, data pre-treatment also known as data correction encompasses data normalisation, centring, scaling, batch effect correction and data integrity checking (Tugizimana *et al.*, 2013; Saccenti *et al.*, 2014; Aksenov *et al.*, 2017). Both pre-processing and pre-treatment contributes to data cleaning to emphasise only relevant biological information (Tugizimana *et al.*, 2013). These steps ultimately determine the quality and quantity of the acquired information and subsequently, the biological knowledge acquired (Aksenov *et al.*, 2017).

Statistical analysis (univariate or multivariate) is an essential step in mining information from the obtained dataset. Univariate data analysis (analysing a single variable) is usually applied to multidimensional data to independently assess the significant variation of metabolites among the different samples analysed (Aksenov *et al.*, 2017; Vargason *et al.*, 2017; Lamichhane *et al.*, 2018). The Student's t-test; ANOVA (analysis of variance) and the Kruskal-Wallis test are all examples of univariate statistical tests (Tan *et al.*, 2016). Alternatively, multivariate data analysis (MVDA) is used to explore and obtain meaningful information by analysing numerous variables simultaneously (Madala *et al.*, 2014; Schillemans *et al.*, 2019). MVDA can be performed through machine learning methods that are either unsupervised or supervised (Saccenti *et al.*, 2014; Aksenov *et al.*, 2017; Lamichhane *et al.*, 2018). Machine learning is a type of artificial intelligence enabling computers to use different algorithms to detect patterns and predict baseline behaviours or properties through training and observation (Cuperlovic-Culf, 2018). In the case of unsupervised learning methods, underlying patterns and trends within the dataset can be identified without detailed or explicit inputs (unlabelled data) from

the user (Sathya, 2013; Cuperlovic-Culf, 2018; Maione *et al.*, 2019). Many unsupervised algorithms have been designed to uncover the complexity of multitudinous datasets. Common examples include principal component analysis (PCA), a linear method, often used to reduce the multi-dimensionality of the dataset; and hierarchical cluster analysis (HCA) which operates based on a distance measured to group data by assessing the similarity and dissimilarity of the observations (Tugizimana *et al.*, 2013; Tan *et al.*, 2016; Maione *et al.*, 2019). Other examples of unsupervised models for dimensionality reduction include locally linear embedding, isomap and independent component analysis (Bunte *et al.*, 2012) and clustering models include K-means (Capó *et al.*, 2018). Subsequently, the supervised methods are applied for regression and classification analyses to evaluate the difference between pre-defined (by the user) classes or groups. These methods include the projection to latent structures-discriminant analysis (PLS-DA), the orthogonal projection to latent structures-discriminant analysis (OPLS-DA), k nearest neighbour (KNN) clustering and more (Sathya *et al.*, 2013; Saccenti *et al.*, 2014; Aksenov *et al.*, 2017; Lamichhane *et al.*, 2018;). Of course, the validation of these models is mandatory and can be carried out using permutation tests and cross-validation methods (Wishart, 2010; Wanichthanarak *et al.*, 2017). The selection of the appropriate chemometric tool is dependent on the aim of the study. Detailed explanations and examples of chemometric models as well as the applications thereof can be obtained from the cited literature.

Although statistical analyses account for existing connections between variables based on their mathematical criteria, it does not take into account any pre-existing correlation originating from the biological origin (Cambiaghi *et al.*, 2017). Thus, it is often recommended to employ several statistical or data mining techniques. Computational and bioinformatics tools and resources for metabolomic analysis have therefore been developed and recently improved significantly. Some of these computational and bioinformatics platforms comprise license and non-license-based resources such as MetaboAnalyst (<https://www.metaboanalyst.ca/>), Metabox omicsX (<https://omictools.com/metabox-tool>), MetaCore omicsX (<https://omictools.com/metabox-tool>), InCroMAP (<http://www.ra.cs.uni-tuebingen.de/software/InCroMAP/index.htm>), SIMCA (soft independent modelling of class analogy, <https://umetrics.com>) and XCMS (<https://xcmsonline.scripps.edu>). Depending on the algorithm packages in these workflows, the nature of data at hand and intended analyses, these tools and resources can be used in combination (Cambiaghi *et al.*, 2017; Wanichthanarak *et al.*, 2017; Lamichhane *et al.*, 2018).

The final step in data mining namely metabolite annotation is critical in metabolomic studies and can happen with or without the statistical analyses step, depending on the biological question (Wishart, 2010). However, for a non-biased assessment, chemometric analyses are required prior and/or after metabolite annotation. Multivariate data analysis (MVDA) techniques are often employed to identify significant ions or signatory markers characterising the biological state(s) or alterations observed at a specific time point under controlled conditions (Wolfender *et al.*, 2013). There are several free libraries and databases that are often used in the annotation of compounds such as PubChem (<https://pubchem.ncbi.nlm.nih.gov/>), Massbank (<https://massbank.eu/MassBank/Search>), PlantCyc (<https://plantcyc.org/>), MetaCyc (<https://metacyc.org/>), Metlin (<https://metlin.scripps.edu/>), KEGG (<https://www.genome.jp/kegg/>), HMDB (<https://hmdb.ca/>), ChEBI (<https://www.ebi.ac.uk/chebi/>), Lipid Maps (<https://www.lipidmaps.org/>), MetaboID (<https://www.ebi.ac.uk/chebi/>) among others (Klassen *et al.*, 2017). Often described as a bottleneck in metabolomics research, metabolite identification remains an unavoidable prerequisite to ultimately analyse the correlation between the identified metabolites and interpret their biological functions within the plant.

2.2.1.4 Biological interpretation of metabolomics data

One of the main objectives of metabolomics studies is to provide an understanding of the biological relevance of identified metabolites under specific conditions. The biological interpretation of metabolomics data relies not only on compound identification but also on functional analysis. As part of functional analysis, mapping and visualisation of identified metabolites on general biological networks / metabolic pathways provide insight into their functions and mechanisms under stated conditions. This can be manually done by summarising the information collected from each metabolite (using literature and databases) into a coherent biological explanation. The manual approach is time-consuming as it focuses on each metabolite individually. Data interpretation is therefore not only restricted to the number of metabolites identified but also to the time necessary for attributing their biological significance. To overcome such limitations, computer-based approaches have been developed over the past years for data interpretation (Cottret *et al.*, 2018; Chong *et al.*, 2019). These tools have been explicitly designed for pathway mapping and biological interpretation such as MetExplore, pathway activity profiling (PAPi), MetaboAnalyst, metabolite pathway enrichment analysis

(MPEA), MeltDB, Meta P-server, MassTriX, BioCyc, Pathos, MetaMapp, visualisation and analysis of networks with related experimental data (VANTED) and TICL, among others. The principal idea behind pathway mapping is the contextual visualisation of metabolomics data which is often time-consuming with regard to metabolite identification (Booth *et al.*, 2013; Chagoyen & Pazos, 2013). Recently, a chemo-enrichment analysis approach has been described by Xia (2017), to overcome the holdup in biological interpretation by directly predicting biological activities from spectral features as well as generating metabolic pathways and networks of all possible metabolite matches. The generated pathways are then compared to pinpoint possible enhanced biological processes within the plant. This approach has the potential to reduce the time and labour associated with metabolite identification and could, in turn, provide great insight into the functioning of the plant, and aid in the identification of agronomically important traits that can be used to construct models for plant breeding studies (Razzaq *et al.*, 2019). Additionally, such innovative approaches could broaden our comprehension of the plant metabolome since our current understanding stems mainly from previous studies done on a handful of model plants, which subsequently confines our knowledge to those particular pathways and limits our understanding of pathways absent in those plants (Hong *et al.*, 2016). The possibilities for metabolomics research, based on the current trend of advancements, are infinite. Expectedly the future in this field could surely provide a great number of constructed metabolic pathways and a more holistic view of plant metabolome.

Plant Metabolites

2.3 Plant Secondary Metabolites

With altering global climates, it has become imperative for organisms to adapt to environmental and biodiversity fluctuations (Thornton *et al.*, 2019). Their survival greatly depends on obtaining resources from their immediate environment to flourish, otherwise, they migrate or relocate. Plants, being sessile organisms, are forced to thrive under constantly changing conditions, therefore plants are often considered the most successful life forms on earth (Delgoda & Murray, 2017). This success is attributed to their ability to survive a variety of abiotic or biotic stresses through physical and biochemical pathways (Simontacchi *et al.*, 2015; Devi *et al.*, 2017; Van Wallendael *et al.*, 2019). The metabolome, being the end product of all biochemical reactions, contains a range of low-molecular-weight molecules (metabolites) that aid in the plant's survival by providing a selective and adaptive advantage to these sessile organisms (Hong *et al.*, 2016). Metabolites are organic compounds involved in numerous biochemical reactions whether it be as reactants, products or intermediates of enzymatic and other chemical reactions occurring within a biological system (Liu & Locasale, 2017). These small molecules can be categorised as primary or secondary metabolites. Generally, the former is directly involved in plant growth, development and reproduction; and includes sugars, lipids, amino acids and intermediates of photosynthesis, energy sources, tricarboxylic (TCA) cycle and glycolysis. The latter on the other hand, is not directly implicated in plant growth, nonetheless is involved in plant-environmental interactions, and particularly in responses to biotic and abiotic stresses and adaptation to environmental factors. As part of the plant defence system, secondary metabolites can be characterised as phytoanticipins, defence metabolites produced and stored constitutively in the plant, or as phytoalexins, defence metabolites synthesised in response to infection (Mazid *et al.*, 2011; Piasecka *et al.*, 2015). Several criteria have been suggested for categorising secondary metabolites such as chemical structure, composition, solubility in organic solvents and the biosynthetic pathways involved. Some of the major groups of secondary metabolites include terpenes, phenolics, alkaloids, flavonoids and saponins (**Figure 2.5**) (Kabera *et al.*, 2014; Pagare *et al.*, 2015; Mera *et al.*, 2019). These metabolite classes are discussed in more detail in the following sections.

2.3.1 Phenolics

Phenols, or phenolics, are a group of secondary metabolites that participate in the development, ecology and physiology of the plant (Lattanzio, 2013). Commonly these metabolites contain one or more phenol group(s) and range from simple structures (one aromatic ring) to highly complex structures (polymeric structures) (Hussein & El-Anssary, 2018). In the plant, phenolics can be present as simple glycosides, ester-linked to cell walls or present as conjugated esters or amides (Al Jitan *et al.*, 2018). These compounds are widespread throughout the plant kingdom and often associated with taste, colour and flavour. Additionally, also valued for their anti-inflammatory, insecticidal, antioxidant and free radical scavenger activities (Shahidi & Ambigaipalan, 2015; Tungmunthum *et al.*, 2018). Based on the chemical structure and origin they can be classified as either simple phenolics, tannins, coumarins, flavonoids, chromones and xanthenes, stilbenes or lignans (Hussein & El-Anssary, 2018). These compounds are synthesised mainly *via* shikimic acid through the phenylpropanoid pathway and/ or the malonate pathway. The shikimic acid pathway leads to the synthesis of phenylalanine, (hydroxy)cinnamic acids and their derivatives (*e.g.* coumarins, lignans and simple phenols among others). Additionally, quinones and xanthenes are synthesised in plant cells through the polyacetate pathway (Mandal *et al.*, 2010; Santos-Sánchez *et al.*, 2019). For simplicity, phenolic acids can be divided into two major groups due to the presence of either hydroxylated derivatives of benzoic acid or cinnamic acid, hence hydroxybenzoic acids and hydroxycinnamic acids (Kumar & Goel, 2019). Hydroxycinnamic acids consist of mainly caffeic -, coumaric -, ferulic - and sinapic acids and are more commonly present than hydroxybenzoic acids which, in turn, consists of derivatives like protocatechuic - vanillic -, syringic - and gallic acids (Taofiq *et al.*, 2017; Bento-Silva *et al.*, 2020). In cereals, phenolics can be found as free phenolics in the outer layer of the pericarp or as bound phenolics which are usually ester-linked to cell walls and only released upon acid, base or enzymatic hydrolysis (Ratnavathi, 2019). In a review by Van Hung (2016) phenolic acid profiles and the antioxidant activity of different cereals were studied; several phytochemicals like phenolics, flavonoids, flavones, and flavanols, anthocyanidins, lignans, and avenanthramides were found among others. The presence of phenolics in these cereals is particularly beneficial to human health in the prevention of chronic diseases like cardiovascular disease, diabetes, and certain types of cancers (Călinoiu and Vodnar, 2018).

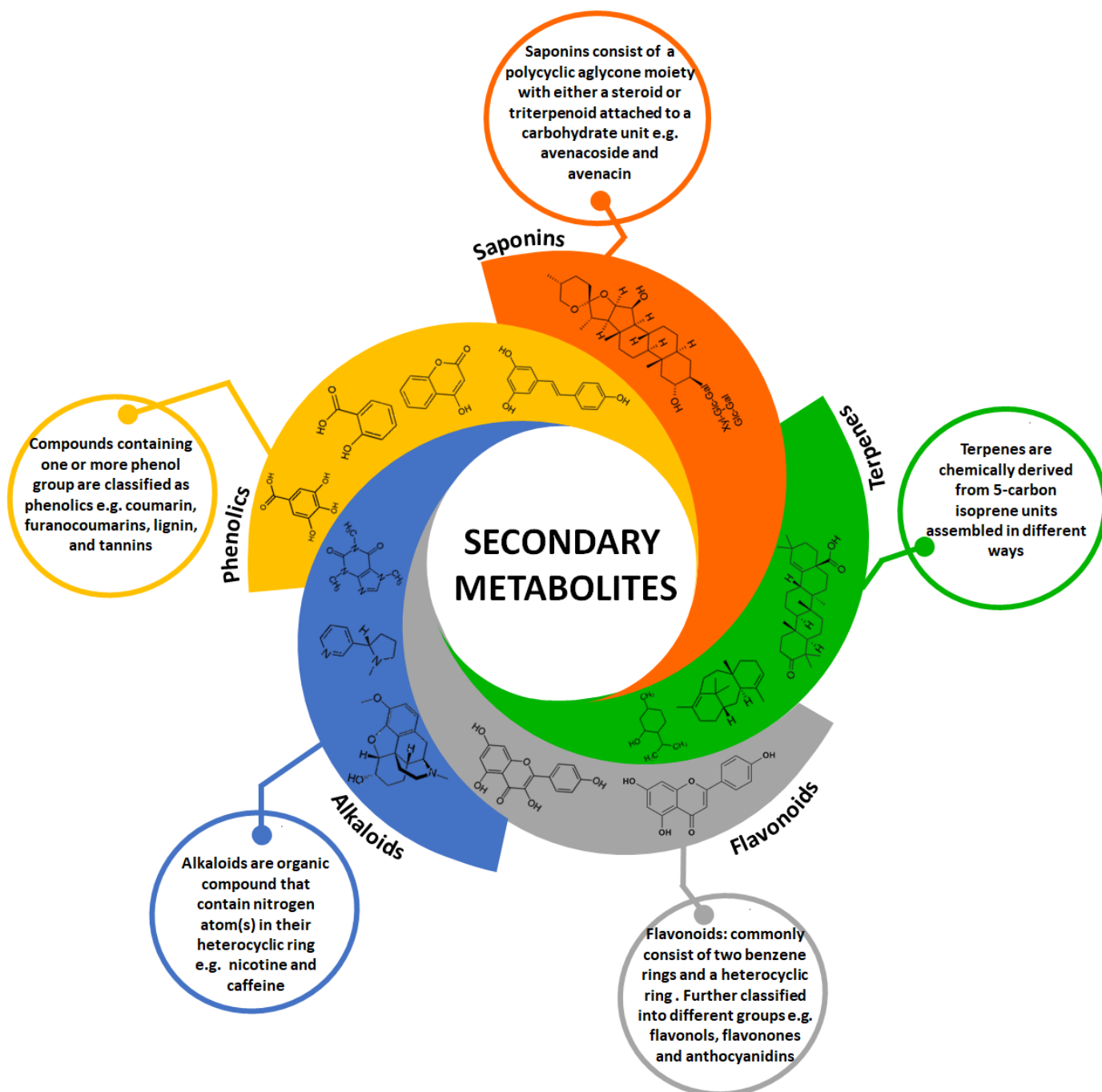


Figure 2.5: Different classes of plant secondary metabolites. These metabolites are diverse and widely spread throughout the plant kingdom, with thousands of different metabolites belonging to each class. Metabolites are commonly grouped based on structural similarities, common functions and biosynthetic pathways.

2.3.2 Alkaloids

Secondary metabolites, being diverse in structure and function, encompass a variety of different compounds with a large group being nitrogen-containing metabolites called alkaloids. These are naturally occurring organic compounds and, as the name suggests, are often alkaline

due to the presence of nitrogen atom(s) (Kaur & Arora, 2015; Peng *et al.*, 2019). Furthermore, the nitrogen atoms also often result in the formation of hydrogen bonds with proteins, enzymes and receptors, thus making these compounds highly bioactive. Alkaloids are often broadly defined due to a lack of homogeneity within these groups, whether it be chemical, biochemical, or physiological, and all differ with the only commonality being that they are all nitrogen-containing (Cushnie *et al.*, 2014; Tok & Gowder, 2019). The classification of these compounds is therefore often based on structure, molecular precursors, biosynthetic origin, the basic heterocyclic nucleus in the structure, pharmacological properties and/ or biological pathways (Eguchi *et al.*, 2019). Generally, alkaloids can be divided into different classes like indoles, aromatics, quinolines, isoquinolines, oxindoles, pyridines and tropanes to name a few (Dey *et al.*, 2020). In plants, alkaloids are often synthesized in response to pathogens or predators to function as defence compounds. Additionally, they have greatly contributed to human health in the form of malarial treatments (*e.g.* quinine) (Achan *et al.*, 2011), asthma relief in the case of ephedrine (Lu *et al.*, 2012), anti-tumour (*e.g.* homoharringtonine) (Lü & Wang, 2014), pain relief, anaesthetics (*e.g.* morphine) (Sayhan *et al.*, 2017), and antibacterial treatments (*e.g.* sanguinarine) (Thawabteh *et al.*, 2019) among others (Bribi, 2018). Furthermore, literature has also reported on improved immune functions, nutrition, and physical performance after the consumption of alkaloid-containing plants such as caffeine (coffee) or guaranine which has shown antioxidant, anti-inflammatory and stimulatory properties. With that said, alkaloids are diverse and not all are beneficial, *i.e.* a range of alkaloids can be harmful or toxic when ingested, depending on the amount consumed, like aconitine, atropine, coniine, colchicine, dimethyltryptamine and suchlike (Diaz, 2015; Matsuura & Fett-Neto, 2015; Adibah & Azzreena, 2019).

2.3.3 Flavonoids

Flavonoids, an important class of plant secondary metabolites, are biosynthetically synthesised from malonyl CoA and *p*-coumaroyl CoA, which are derived from the acetate and shikimate pathways, respectively (Yonekura-Sakakibara *et al.*, 2019). They are a large group of phenolic derivatives characterised by a core structure composed of two phenolic rings connected by three carbon units (Kumar & Pandey, 2013). Here, they are discussed due to the numerous structural varieties and biological functions. These include functioning as signal molecules, allelopathic compounds, phytoalexins, antioxidants, antimicrobials and as a range of defence

compounds against biotic and abiotic stresses (Panche *et al.*, 2016). Additionally, flavonoids have been used extensively for pharmaceutical and medicinal purposes to promote human health as antioxidants, anti-inflammatories, anti-mutagenic, and anti-carcinogens, among others (Tungmunthum *et al.*, 2018). Different subgroups of flavonoids are known to have different functions and subdivided based on structural properties. These subgroups include anthocyanins, chalcones, flavanones, flavones, flavonols and isoflavonoids (**Figure 2.6**).

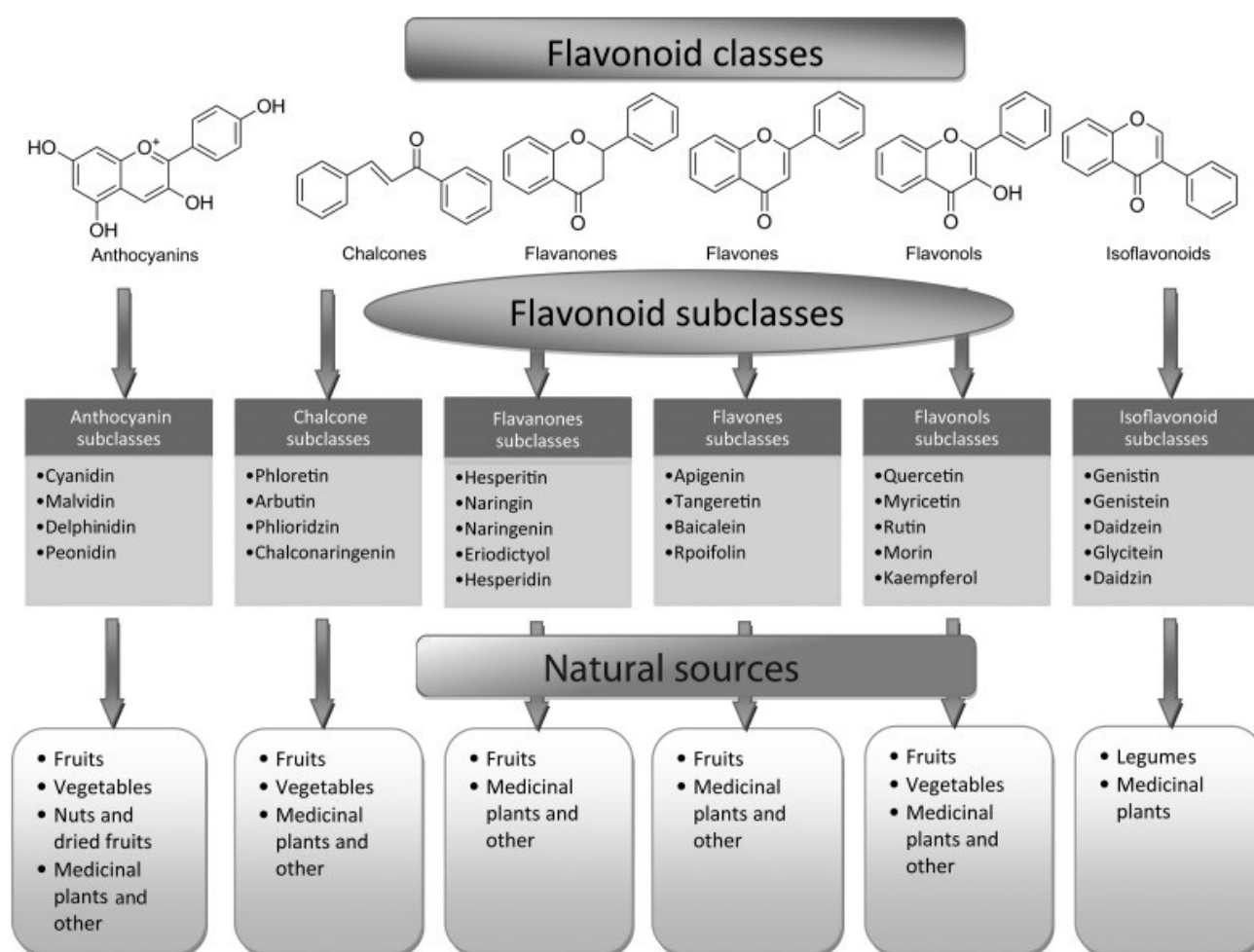


Figure 2.6: The different flavonoid classes. Shown are their core structures, subclasses and natural sources (Panche *et al.*, 2016).

2.3.4 Terpenes

Terpenes are a large group of hydrocarbon molecules that are derived from multiple condensations of five carbon (isoprene) units (Tetali, 2019). The number of 5-carbon units is used to distinguish between the various terpenes like monoterpenes (C₁₀), sesquiterpenes (C₁₅), diterpenes (C₂₀), sesterterpenes (C₂₅), triterpenes (C₃₀), tetraterpenes (C₄₀), and polyterpenes (>C₄₀) (Singh & Sharma, 2015). These compounds are synthesised by a range of organisms and are diverse in complexity and biological activities (Mugford *et al.*, 2012). In the plant kingdom, they have various roles as defence compounds, plant hormones, vitamins, electron carriers, and membrane components. They are also highly aromatic, giving plants their signatory flavours and scents which are often produced to attract (pollinators) or repel (predators) insects, thus ensuring the survival of the plant (Abbas *et al.*, 2017). Complex terpenes (several isoprene units) function as precursors for bioactive molecules like cholesterol and steroidal hormones that have a role as protective layers in plants. Due to the various biological properties, terpenes have also gained increasing popularity for use in pharmaceuticals, foods and cosmetics (Paduch *et al.*, 2007; Cox-Georgian *et al.*, 2019). These compounds are synthesised *via* mevalonic acid (cytosol) and methylerythritol phosphate (chloroplast) pathways, both of which produce isopentenyl diphosphate (IPP). IPP is the central intermediate in the biosynthesis of isoprenoids and is, therefore, an important precursor in the isoprenoid pathway. A large variety of terpenes have been identified in the plant kingdom. In general, these compounds have been found to have various health-promoting effects such as anti-inflammatory, antioxidant, antidepressant, and even anti-cancer properties in some cases (Rogerio *et al.*, 2009; Rufino *et al.*, 2015; Yu *et al.*, 2017^a; Cox-Georgian *et al.*, 2019).

2.3.5 Saponins

As secondary metabolites, saponins are described as a structurally complex class of amphipathic glycosylated triterpenoids, steroids and steroidal alkaloids. A range of ecological functions have been associated with these compounds including plant defence where cytotoxic, hemolytic, molluscicidal, anti-inflammatory, antifungal, anti-yeast, antibacterial and antiviral activities are commonly seen, and additionally found to function as allelopathic agents between competing plants (Arabski *et al.*, 2012; Moses *et al.*, 2014; Hussein & El-Anssary, 2018). Moreover, some saponins have pharmacological roles that include anti-carcinogenic, anti-

microbial, cholesterol decreasing, immune-modulating, as well as anti-inflammatory properties (Moses *et al.*, 2014; Barbosa, 2014). Most monocotyledonous plants do not contain saponins, with oat being the exception. Here, these phytoanticipins possess a range of activities that aid in the defence against pathogens or other environmental threats. Two types of saponins are commonly and uniquely found in oat – avenacins (triterpenoid saponins) and avenacosides (steroidal saponins) (see section 2.3.6). As the only saponin-accumulating cereal, oat has been extensively studied regarding the presence of these saponins (Mylona *et al.*, 2008; Pecio *et al.*, 2013; Yang *et al.*, 2016; Hu & Sang, 2020). Saponin activity seems to be strongly related to the structural properties that allow them to self-aggregate or to form complexes with membrane components such as cholesterol and phospholipids (El Barky *et al.*, 2017). Structurally, saponins contain amphiphilic groups that usually consist of one or more hydrophilic sugar component(s) and a lipophilic steroid or triterpenoid component(s) (Lorent *et al.*, 2014). Saponins are thus diverse due to the presence of different sugars (*e.g.* glucose, rhamnose, galactose, glucuronic acid, arabinose, xylose and fructose), sugar branches, and sapogenins. They can therefore be classified as monodesmosidic (single sugar), bidesmosidic (two sugar chains) and polydesmosidic (multiple sugar chains) according to the number of sugar chains they contain (Scognamiglio *et al.*, 2015; Tatia *et al.*, 2019).

2.3.6 Secondary metabolites in oat

Oat synthesises a variety of phytochemicals with high antioxidant -, anti-inflammatory and antiproliferative activities. Among these are saponins (Yang *et al.*, 2016), phenolics (Dimberg *et al.*, 2005), flavonoids (Bahraminejad *et al.*, 2008), avenanthramides (de Bruijn *et al.*, 2019), tocopherols and tocotrienols (Peterson *et al.*, 2007; Gutierrez-Gonzalez *et al.*, 2013). As previously mentioned, oat is the only saponin-accumulating cereal which synthesises two unique saponins known as avenacins and avenacosides (Osbourn, 2003), and are synthesised *via* two separate biosynthetic pathways from the condensation of either a triterpenoid or steroid moiety with one or more glycosyl unit(s) (Moses *et al.*, 2014). Avenacoside A and B are the two primary steroidal glycosides that accumulate primarily in the leaves and grains of oat. They are glycosylated at the C-3 with single rhamnose and two glucose units (avenacoside A) and in the case of avenacoside B, rhamnose, and three glucose units (**Figure 2.7**) (Pecio *et al.*, 2012; Yang *et al.*, 2016). These preformed inactive glycosylated saponins (phytoanticipins) are stored in the vacuole of the plant. Upon exposure to pathogens, the tissue membrane becomes

disrupted, allowing these compounds to become active through hydrolysis with a β -glucosidase enzyme known as avenacosidase. This enzyme cleaves the D-glucose unit resulting in a biologically active aglycone known as 26-deglucoavenacoside (A or B depending on the sugar moieties). The remaining sugar moieties attached at the C-3 sites are essential in combating pathogens by allowing the active avenacoside to complex with membrane sterols, thereby disrupting the plasma membrane and resulting in cell death (Morrissey *et al.*, 2000; Mylona *et al.*, 2008; Piasecka *et al.*, 2015; Doligalska *et al.*, 2017). Avenacins are a uniquely-produced group of triterpenoid saponins found in the roots of oat plants. These compounds are also preformed and stored in the plant vacuole, but in contrast to avenacosides, they are readily present in active, glycosylated forms (Osbourn, 2003; Piasecka *et al.*, 2015). These triterpenoid saponins are synthesised from mevalonic acid through the isoprenoid pathway and ultimately stored for defence against root-infiltrating pathogens. Four avenacins have been identified namely A-1, A-2, B-1 and B-2. These function as antifungal defence compounds, similar to the avenacosides, by forming complexes with membrane sterols and thereby creating pores in the membrane (Mugford & Osbourn, 2012; Kazan & Gardiner, 2017).

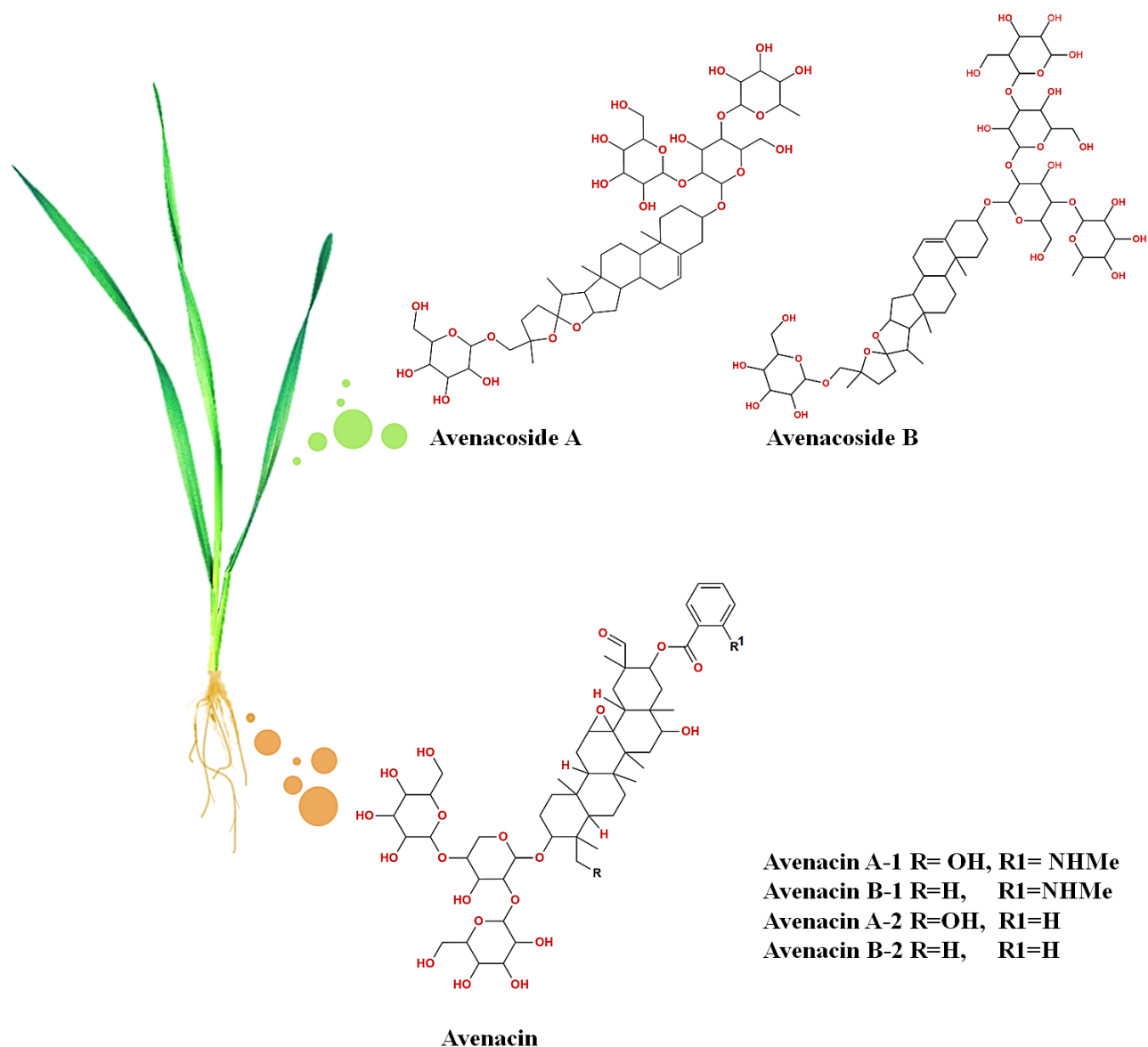


Figure 2.7: Structures of the major oat saponins. The structures, as described in the text, of two steroidal saponins found in the leaves of oat namely avenacoside A (one rhamnose and two glucose units) and avenacoside B (one rhamnose and three glucose units), and four triterpenoid saponins found in oat roots, avenacins A-1, B-1, A-2 and B-2.

Avenanthramides (Avns) are low-molecular-weight compounds that are uniquely present in oat. They have been known to function as phytoalexins with antimicrobial or antifungal activities (Okazaki *et al.*, 2004; Perrelli *et al.*, 2018). Additionally, they can become incorporated into the cell wall upon infection to offer reinforcement against degradation which often occurs due to the enzymes secreted by pathogens (Ishihara *et al.*, 2014). More than thirty different types have been identified in leaves and grains, with avenanthramide A, B and C being the most abundant (**Figure 2.8**) (Meydani, 2009).

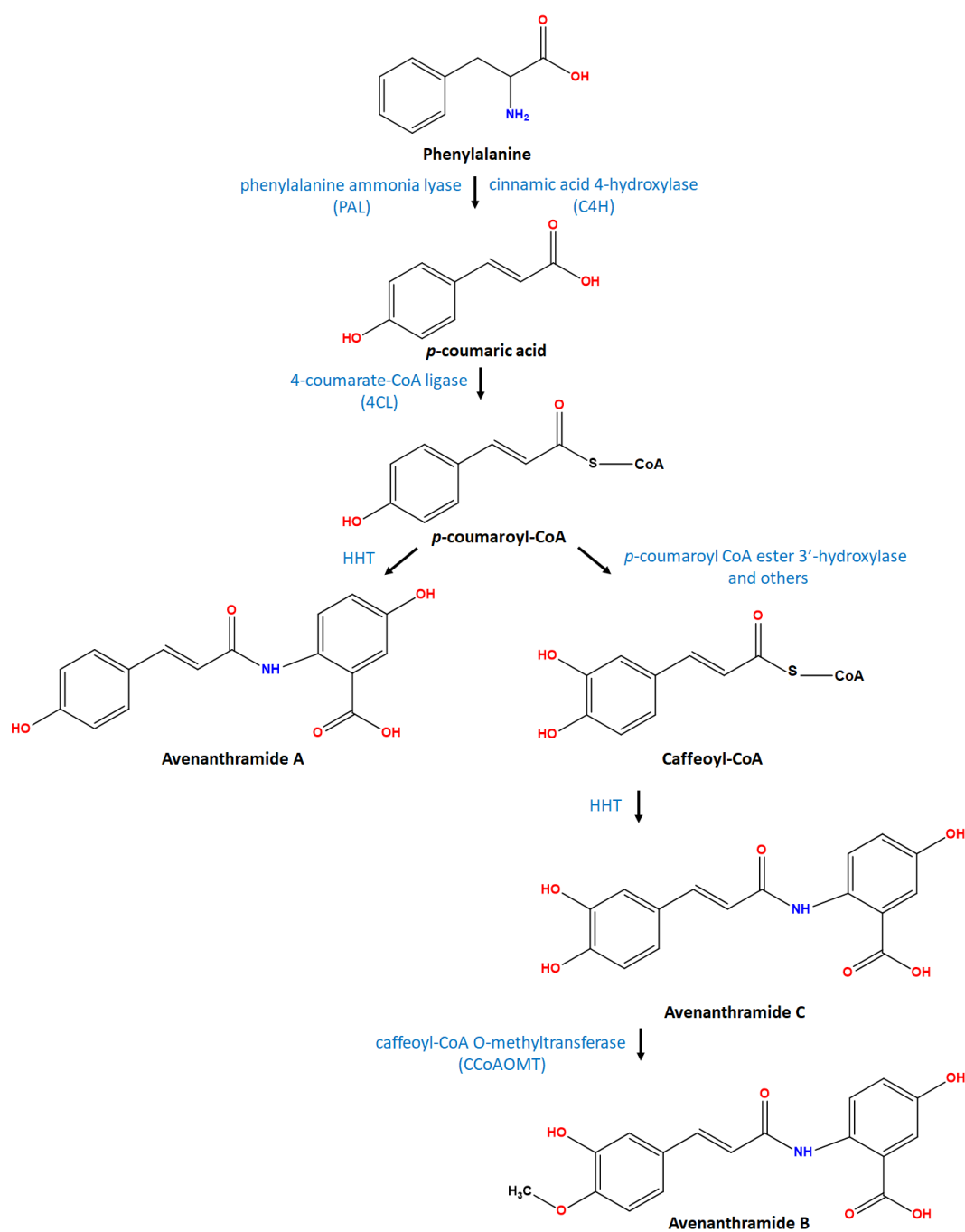


Figure 2.8: The biosynthesis pathway illustrating the formation of the three major avenanthramides in oat (adapted from Li *et al.*, 2019).

These Avns are derived from α -pyrrolidone and structurally consist of 5-hydroxyanthranilic acid with *p*-coumaric -, ferulic -, and caffeic hydroxycinnamic acids, respectively (Li *et al.*, 2019). The biosynthesis of these major avenanthramides (A, B and C) occur through the condensation of a hydroxyanthranilic acid with hydroxycinnamoyl-CoA and its derivatives

(Ishihara *et al.*, 2014). This process follows multiple reactions catalysed by multiple enzymes, with the main enzyme being hydroxycinnamoyl-CoA: hydroxyanthranilate N-hydroxycinnamoyltransferase (HHT). The biosynthesis of these compounds is further described in this section. The process is initialised by the synthesis of *p*-coumaric acid from phenylalanine in the presence of phenylalanine ammonia lyase (PAL) and cinnamic acid 4-hydroxylase (C4'H). The derived *p*-coumaric acid is then subsequently transformed into its activated CoA thioester counterpart by 4-coumarate-CoA ligase (4CL), to condense with 5-hydroxyanthranilic acid, catalysed by HHT, to form avenanthramide A. Conversely, the *p*-coumaroyl-CoA is often converted to *p*-coumaroyl shikimate or quinate first, in which case it becomes hydroxylated by *p*-coumaroyl CoA ester 3'-hydroxylase to form caffeoyl-CoA. Subsequently, the caffeoyl-CoA is then condensed with the 5-hydroxyanthranilic acid in the presence of HHT to form avenanthramide C. Finally, avenanthramide C is methylated by the caffeoyl-CoA O-methyltransferase enzyme to form avenanthramide B (**Figure 2.8**) (Matsukawa *et al.*, 2002; Yang *et al.*, 2004; Li *et al.*, 2019). The formation and synthesis of these compounds have gained increasing interest due to numerous health-promoting benefits as previously mentioned (Meydani, 2009; Tripathi *et al.*, 2018; Fu *et al.*, 2019; Turrini *et al.*, 2019).

Plant Breeding

2.4 Plant Breeding and Crop Improvement

Plant breeding can be described as the deliberate manipulation of plant attributes to enhance specific traits and is therefore important for improving crop yield potential and agronomic characteristics in plants. This technology dates back to the first domestication of plants, where unintentional changes occurred with agricultural domestication and later intentional changes through molecular-based tools (Tiwari, 2017). In any case, plant breeding is driven by the need to improve plant characteristics through the creation of desired genotypes and phenotypes in new cultivars. Plant varieties and cultivars have resulted from the domestication of ancestral wild plant species. Since domestication, modern plants have lost valuable traits due to the reduction of genetic diversity. The limited adaptability of crops to the ever-changing environment, climate change, and increasing food demands have resulted in growing pressure on plant breeders. Fortunately, many important traits, particularly those associated with abiotic and biotic resistance, are still conserved in wild relatives of crops such as rice, maize, wheat, barley, and oat (Zhang *et al.*, 2017^a; Mammadov *et al.*, 2018). Therefore, breeders are increasingly using wild relatives, with a process known as pre-breeding, to reintroduce some of these traits and enhance genetic diversity. The insertion of wild relative traits into modern cultivars can be achieved by conventional breeding or by molecular breeding technologies (Dempewolf *et al.*, 2017; Zhang *et al.*, 2017^a; Mammadov *et al.*, 2018). With the breakthrough of Mendel's work in 1900 on the law of inheritance, the scientific basis of the technology was established (Duvick, 1996). This led to the modern era of plant breeding which has evolved tremendously since then. Advancements involved the introduction of different techniques includes double haploid technology, hybrid breeding, wild crosses, introgression of traits from wild relatives, embryo and ovule rescue, mutagenesis, protoplast, and plant cell/tissue/organs cultures and regeneration (Pilacinski *et al.*, 2011; Lusser *et al.*, 2012).

Oat plant breeding programs have been aimed at maximising yields, however, many different factors may influence crop yields such as pests, diseases, and environmental factors. Susceptibility to these influences can affect both the yield and quality and therefore finding 'ideal' characteristics in crop varieties are essential (Tester & Langridge, 2010). In this regard,

the identification, tolerance, and susceptibility abilities of various cultivars become an extremely important aspect in agricultural systems (Korir *et al.*, 2013). The identification of cultivars and varieties is not only essential for finding favourable traits but are also extremely important steps during plant breeding, registration, trade, inspection, and seed production. A rapid and effective fingerprinting method is therefore required for early cultivar identification which is important for the protection of breeders' intellectual property rights (Xu & Crouch, 2008; Korir *et al.*, 2013). To date, studies in plant breeding have employed marker-assisted selection (MAS) for cultivar identification and crop improvement. The selection involves biochemical-, morphological-, cytological- and DNA-based markers (Roychowdhury *et al.*, 2013). Since the development of molecular markers, key challenges in conventional crop improvement programs have been addressed (Prohens, 2011). The selection of desired traits in plant breeding is an example of such a challenge, although the presence of a gene does not always imply its full expression (Cobb *et al.*, 2019). The plant metabolome is a representation of the phenotype, and therefore the study of the metabolome, metabolomics, becomes a valuable tool for the rapid detection of new traits, the identification of cultivars and differentiation among cultivars (Hall, 2006).

2.4.1 Metabolomics for cultivar identification in plant breeding

Metabolomics has the potential to provide new tools to plant breeders for cultivar identification and evaluation of agronomic traits. When complemented by genomic studies it could result in genetic improvement in crops based on chemical composition. Improvements could be nutritional, functional, or in the resistance of the crop species to numerous factors (Kumar *et al.*, 2017). The integration of metabolomics with genomics, transcriptomics, proteomics and genetic modification has been applied in crop yield and quality improvement studies. Furthermore, in crop breeding, it has been applied for the selection of agronomically important traits thereby aiding in the improvement of cultivars and varieties (Simó *et al.*, 2014; Hong *et al.*, 2016; Sharma *et al.*, 2018). The concept of applying metabolomics for grain crop breeding studies was previously explored (Ferne & Shauer, 2009). Although metabolomics was, at the time, still an emerging field, a cost-effective integration with genome sequencing allowed its application in crop breeding programs. These earlier studies paved the way for metabolomics in plant breeding, and in recent reviews (Kumar *et al.*, 2017; Alseekh *et al.*, 2018), it is apparent just how much metabolomics research has progressed to its current position. These reviews

elaborated on studies that pointed out how metabolomics had enabled the selection of a greater number of desirable traits through technological advancements, allowed the construction of improved metabolic networks, and identified biomarkers for unravelling function and contribution toward improving plant yield, quality and shelf life. As we now know, the emergence of metabolomics is highly promising for the prediction of a variety of agronomically important phenotypes and particularly for discovering signature metabolites or metabolic markers (biomarkers) linked to traits of interest (Zabotina, 2013; Sharma *et al.*, 2018). Metabolomics has been particularly useful in the identification and differentiation of grain crops (Hamany Djande *et al.*, 2020), and with technological advancements, the potential of metabolomics becomes endless.

Plant Defence

2.5 Plant Defence

Plants have an innate immune system that functions through several surveillance-type receptors that allow the detection of pathogens and subsequently activates signal transmission to promote optimal defence responses to the attack (Andolfo & Ercolano, 2015). Due to the sessile nature and the absence of an established circulatory immune system, plants have developed a range of adaptive changes at a transcriptional and post-transcriptional level to combat different stresses (Gull *et al.*, 2019). In the environment plants are exposed to both abiotic (*e.g.* drought, climate change, floods, radiation and wind) and biotic stresses (*e.g.* bacteria, viruses, fungi and insects). These stresses could impair plant health, and subsequently plant functioning and reproduction (Elad & Pertot, 2014). To deal with these stresses plants have developed an advanced immune system that includes innate immunity and inducible responses such as (i) systemic acquired resistance (SAR), (ii) induced systemic resistance (ISR) and (iii) priming (Tugizimana *et al.*, 2018). As the first line of defence, plants have an intact waxy cuticle that acts as a barrier to protect against pests and pathogens. These exterior barriers can, however, be compromised, thus allowing pathogens to enter the plant (War *et al.*, 2012). Once this happens, the plant must resort to a range of different defence mechanisms that ultimately lead to the production of secondary metabolites, antimicrobial compounds and defensive proteins (González-Lamothe *et al.*, 2009). The perception and optimal response of plants depend on various receptors that recognise and respond to extracellular signals, such as pattern recognition receptors (PRRs), and in the case of intracellular signals, nucleotide-binding leucine-rich repeat (NB-LRR) proteins, with the former being a basal defence response. This type of first-line immunity is triggered by a microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) which are essential structures conserved in microbes and pathogens, and are therefore responsible for triggering either MTI (MAMP-triggered immunity) or PTI (PAMP-triggered immunity). Additionally, plant-feeding insects elicit a similar response perceived by PRRs by producing herbivore-associated elicitors (HAEs), herbivore-associated molecular patterns (HAMPs) or effectors that trigger defence responses. The latter is carried out by plant resistance proteins that are equipped to recognise specific effectors, either directly by physical association or indirectly through an accessory protein, such as avirulence (Avr) proteins which

subsequently activate effector-triggered immunity (ETI) (Jones & Dangl, 2006; Bonaventure *et al.*, 2011; Choi & Klessig, 2016; Miller *et al.*, 2017). Activation of ETI induces various defence mechanisms, such as SAR, ISR and priming. SAR is a form of induced resistance that is activated after a plant is exposed to various virulent elicitors and subsequently leads to the accumulation of SA to stimulate defence mechanisms. In severe cases, a hypersensitive response (HR) can also be triggered and lead to apoptosis (programmed cell death) of infected - and surrounding cells. ISR, on the other hand, stimulates defence through the jasmonate and ethylene pathways often in response to beneficial microbes like growth-promoting fungi. Another inducible state of defence has been described, where the plant is primed to respond rapidly and efficiently to various stresses, and this induced state is called priming and can result from SAR, ISR or other inducible defence mechanisms (Conrath, 2006; Pieterse *et al.*, 2014; Mauch-Mani *et al.*, 2017; Tugizimana *et al.*, 2018). These induced defences ultimately lead to the production of secondary metabolites, antimicrobial compounds, proteins and antimicrobial enzymes (González-Lamothe *et al.*, 2009). Ultimately, metabolomic studies regarding plant stress responses could result in a better understanding of the plants' phenotypic plasticity as well as their resistance or tolerance mechanisms that could subsequently be applied in plant breeding (Genga *et al.*, 2011; Tian *et al.*, 2016).

2.5.1 Plant response to abiotic stresses

Stress-inducing agents, referred to as external conditions that exhibit an adverse effect on plant growth, development and reproduction, can activate a range of plant responses to counteract detrimental effects due to the abiotic – or biotic stressors (Gull *et al.*, 2019). Since the world population has been rapidly increasing, a corresponding increase in food demand has to be met. With sudden changes in environmental conditions, harsh impacts are observed on crops not able to tolerate these stresses and are subsequently impaired, thereby resulting in yield losses. With the ever-changing global climates, the intensity of abiotic stress is predicted to increase. Hence the development of crop varieties that are tolerant to abiotic stresses to ensure food security in coming years are essential (Raza *et al.*, 2019; Ritchie & Roser, 2020). Under abiotic stress conditions, plants respond by carefully and rapidly allocating resources and energy toward fending off the stress and subsequently preventing further exposure to opportunistic stresses (Rejeb *et al.*, 2014). When exposed to various primary stresses (salinity, cold, drought, heat, radiation, and/ or chemical pollution), several secondary changes occur, such as stomatal

closure, imbalanced water, and nutrient uptake, impaired photosynthetic functioning, altered gaseous exchange, and the generation of reactive oxygen species (ROS) along with a range of signalling phytohormones such as abscisic (ABA), ethylene (ET), jasmonic acid (JA), and salicylic acid (SA) (Sharma *et al.*, 2012; Tiwari *et al.*, 2017; Xie *et al.*, 2019). The integrative effect these factors induces oxidative damage to DNA, proteins, lipids and carbohydrates causing changes in the redox, osmotic, ionic and energetic stability of the plant. Subsequently, these changes initiate a cascade of events to restore homeostasis, such as downstream signalling processes and gene activation through transcription factors (Sharma *et al.*, 2012; Hotamisligil & Davis, 2016). Stress response mechanisms are initiated and involve restoring osmotic balance through the synthesis of osmolytes (Brocker *et al.*, 2012), ion transport to restore ionic homeostasis (Conde *et al.*, 2011), enzymatic and nonenzymatic antioxidants are produced for cellular detoxification of ROS (Birben *et al.*, 2012), activation of chaperones to restore damaged proteins (Voth & Jacob, 2017), and maintenance of redox and energetics. These coordinated systems result in re-established cellular homeostasis, protection of functional and structural proteins and membranes, and ultimately the tolerance to abiotic stresses (Duque *et al.*, 2013; He *et al.*, 2018) (**Figure 2.9**). Insufficient responses at any of the steps in signalling and gene activation may cause irreversible changes and the destruction of functional as well as structural proteins and membranes, subsequently resulting in cell death (Wang *et al.*, 2003).

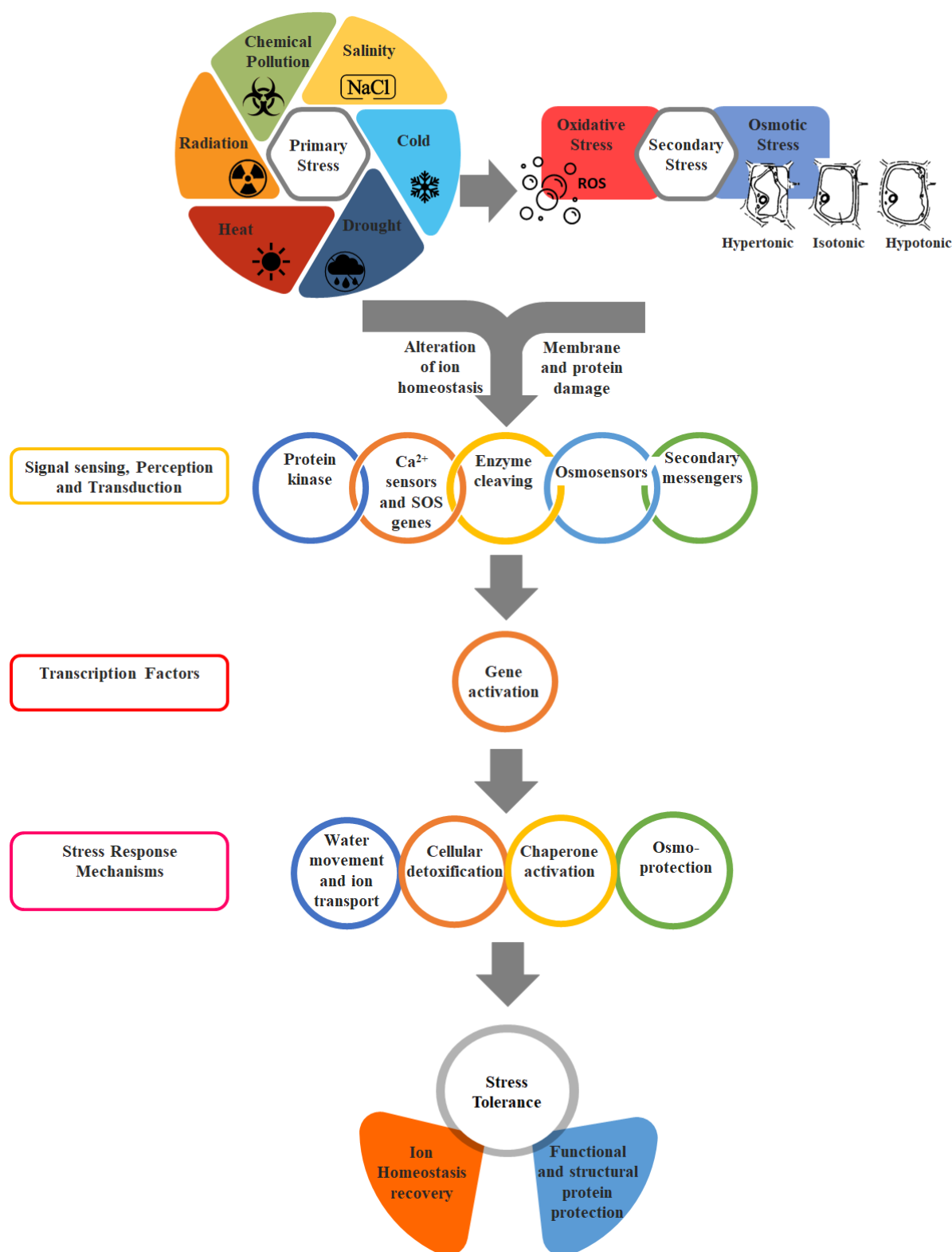


Figure 2.9: Schematic representation showing the complexity of the plant response to abiotic stress and mechanisms of stress tolerance. Primary stresses, such as drought, salinity, cold, heat and chemical pollution are often interconnected, and cause cellular damage and secondary stresses such as oxidative and osmotic stress. These stress signals trigger the downstream signalling process and transcription controls which activate stress-responsive mechanisms to restore homeostasis. Inadequate response to these stresses may result in irreversible damages and destruction of functional and structural proteins and membranes, resulting in cell death. (Figure was compiled and adapted from various sources to summarise different aspects of plant stress; Wang *et al.*, 2003; Duque *et al.*, 2013; Patel *et al.*, 2019).

2.5.2 Plant response to biotic stresses

Pathogens have used plants as a source of nutrients for many years, subsequently leading to the evolution of plants to develop various immune responses to combat infection caused by these microorganisms (Abramovitch *et al.*, 2006). When plants are exposed to pathogens, two possible outcomes can be described namely a compatible- or incompatible interaction. Compatible interactions occur when the pathogen successfully infects the plant, *i.e.* the pathogen is virulent and the plant susceptible, conversely, the opposite is true for incompatible interactions where the plant successfully wards off the pathogen and avoids infection, *i.e.* the pathogen is avirulent and the plant resistant (Gupta *et al.*, 2015; Ponzio *et al.*, 2016). To deal with biotic stresses plants have developed an advanced immune system. Other than the physical barriers previously mentioned (waxes and thick cuticles), two defence responses are triggered when these barriers fail to prevent the pathogen from entering the plant. As such, M/PTI and ETI can be described as the initial response mechanisms activated upon pathogen infection (Gimenez-Ibanez *et al.*, 2018). These defences are triggered upon recognition of pathogenic features such as MAMPs or PAMPs and results in the reinforcement of plant cells in response (Hein *et al.*, 2009; Dalio *et al.*, 2017). On the other hand, pathogens have also developed countermeasures to suppress innate immunity, in this case, the plant responds with a subsequent defence mechanism known as the hypersensitive response (HR), often triggered by ETI. This type of induced response is characterised by programmed cell death as an attempt to inhibit the pathogen from infecting neighbouring cells. Once triggered, plants develop a heightened state of defence for a certain period of time, known as SAR (Balakireva & Zamyatin, 2018; Balint-Kurti, 2019). These mechanisms are described in more detail in the sections below.

2.5.2.1 M/PAMP- and effector-triggered immunity (M/PTI & ETI)

M/PTI is triggered by the perception of M/PAMPs through plasma membrane-bound extracellular receptors, called pattern recognition receptors (PRRs) that include receptor-like kinases (RLKs) and receptor-like proteins (RLPs). M/PAMPs are essential components, conserved throughout various pathogen classes and often include lipoproteins, ergosterol, flagellin, peptidoglycans, xylanase, chitin, and lipopolysaccharides (LPS), among others (Mackey & McFall, 2006; Mogensen, 2009; Newman *et al.*, 2013). The perception and

activation of defence mechanisms in response to different M/PAMPs are often conserved in different plant species (specific to each plant species). Conversely, some M/PAMPs (*e.g.* chitin, LPS, and flagellin) are recognised by a range of host plants and subsequently trigger corresponding immune responses (Newman *et al.*, 2013; Nishad *et al.*, 2020). In the event that these M/PAMPs are perceived, M/PTI is activated and signals the closure of the stomata to limit pathogen spread, produces reactive oxygen species (ROS) and nitric oxide (NO), limits nutrient transfer from the cytosol to the apoplast, causes callose deposition, and synthesises antimicrobial metabolites and defence hormones (Bigeard *et al.*, 2015; Lu & Yao, 2018). A common example of M/PTI response triggered by bacterial-derived flagellin peptide (flg22) has been extensively studied. The flg22 M/PAMP has been known to elicit an M/PTI response through recognition by the RLK, FLS2 (receptor-like kinase flagellin sensing 2), complex with BAK1 (BRI1-associated kinase) regulatory kinase. This specific binding occurs between flg22 and FLS2 which subsequently activates the FLS2-BAK1 complex and leads to the activation of M/PTI (Yu *et al.*, 2017^b). Soon after recognition, a signalling cascade is triggered that leads to elevated levels of calcium ions, ROS, activated kinases like cyclin-dependent kinases (CDKs), and mitogen activated protein kinases (MAPKs). Consecutive changes also occur in cellular proteins and gene regulation to initiate the synthesis of various anti-microbial agents (Taj *et al.*, 2010).

In addition to M/PAMPs, plants can also respond to various endogenous elicitors that are produced due to cell damage by lytic enzymes synthesised by pathogens to break open cells for entry into the plant. These degraded products (*e.g.* cell wall fragments) thus also serve as elicitors called damage-associated molecular patterns (DAMPs). These compounds are secreted from dying cells due to damage, trauma, or necrosis, and can additionally be released from immune, or severely stressed cells. Although DAMPs are derived from host cells, in contrast to M/PAMPs which are pathogen-derived, they both initiate innate immune responses (Shaefer, 2014; Choi & Klessig, 2016; Hou *et al.*, 2019). Relatedly, plants have also adapted a range of defence strategies against herbivory, and these responses depend on the recognition of herbivore-associated molecular patterns (HAMPs). HAMPs is similar to M/PAMPs and are produced by insects during feeding in the form of saliva, oral secretions (OS), and digestive waste products among others. Additionally, wound signals are also perceived upon feeding and contribute to the activation of defence responses. Herbivory has also been known to affect stress hormones, secondary metabolites, defence proteins, resource allocation, leaf maturation and ultimately suppresses or activates plant defence (Mithöfer & Boland, 2008; War *et al.*,

2012). Various stress hormones have been reported in plant defence against herbivores and among these, JA has been known to play an essential role in activating direct and indirect defence responses. Additionally, the JA signalling pathway is also involved in crosstalk networks with other phytohormone signalling pathways such as ET, SA, and ABA. These signalling responses initiate important downstream defence responses such as the production of α -amylase inhibitors, lectins, chitinases, and polyphenoloxidases that function as defence proteins to reduce the insect's capacity to digest the plant material and subsequently reduce feeding (Erb *et al.*, 2012; War *et al.*, 2012).

Pathogens have developed countermeasures to suppress plant defences, which involve the production of effector proteins often referred to as avirulence (Avr) factors. Subsequently, plants have responded by employing receptors to recognise these effectors, such as NB-LRRs, to subsequently activate ETI. ETI has therefore been considered a rapidly amplified state of defence compared to PTI (Thomma *et al.*, 2011; Miller *et al.*, 2017). Phytohormones or signalling molecules, among other factors, have been known to be essential in plant development, regulating cellular processes, and signalling plant defence responses. The regulation and production of these phytohormones are thus imperative for plant survival, especially during plant-pathogen interactions. Three major plant stress hormones have been reported known as JA, SA, and ET. Other hormones such as ABA, auxin, cytokinin (CK), gibberellic acid (GA), and peptide hormones have also been reported as regulators of plant defence. JA and ET are commonly involved in regulating defence against necrotrophic pathogens (pathogens that kill the host) and insects, whereas, SA has played a significant role in the defence against biotrophic pathogens (pathogens that depend on the host for survival) (Bari & Jones, 2009; Zhang *et al.*, 2017^b). Although these hormones are responsible for regulating distinct pathogen-related genes, the crosstalk between them plays an important role in inducing plant defence (Kouzai *et al.*, 2018). Effectors not only trigger defence responses locally, but they also induce the production of signals such as SA and methyl salicylic acid (MeSA), among others to activate systemic expression of antimicrobial pathogenesis-related (PR) genes that produce PR proteins in areas surrounding the site of infection to protect the plant from developing a secondary infection (Gao *et al.*, 2015). This is known as SAR, which in a nutshell is an enhanced state of resistance that occurs when plants have been primed previously by exposure to the pathogen and thus respond more effectively to subsequent infections. This type of systemic resistance can last for long periods of time ranging from weeks to months and in some cases even the whole growing season (Fu & Dong, 2013). Induced

systemic resistance (ISR) is another inducible plant defence response that can be differentiated from SAR with regard to the type of elicitor it responds to and the regulatory pathways involved. ISR is initiated by the recognition of plant growth-promoting bacteria (PGPB), and contrary to SAR, does not result in the production of PR proteins or SA; instead, ISR relies on JA and ET pathways to regulate responses (Heil & Bostock, 2002; Pieterse *et al.*, 2014). Both the salicylate- and jasmonate-induced pathways are equally important in the plant defence as they lead to the production of PR proteins (antifungals) and oxidative enzymes (peroxidases), respectively. Additionally, they also activate the accumulation of antimicrobial compounds known as phytoalexins (Ahuja *et al.*, 2012; Ali *et al.*, 2018). As mentioned in **section 2.3**, phytoalexins are defence metabolites (secondary metabolites) synthesised in response to infection to function as antimicrobial and antioxidative substances.

2.5.2.2 Zig-Zag model of plant innate immunity

The overall outcome of plant-pathogen interactions depends on how the plant responds to the pathogen and how the pathogen, in turn, counteracts this response. The Zig-Zag model was proposed by Jones & Dangl (2006) to describe plant-pathogen interaction mechanisms. Initially, M/PTI-based defences are triggered upon the recognition of M/PAMPs by PRRs, to limit pathogen growth. This initial response is subsequently suppressed by successful pathogens after the production of effectors to further promote pathogen growth, which is referred to as effector-triggered susceptibility (ETS). NB-LRR-based recognition occurs as a countermeasure by the plant and results in the activation of ETI. Multiple rounds of ETS and ETI can occur, with the outcome often depending on the amplitude of disease resistance or susceptibility (**Figure 2.10**).

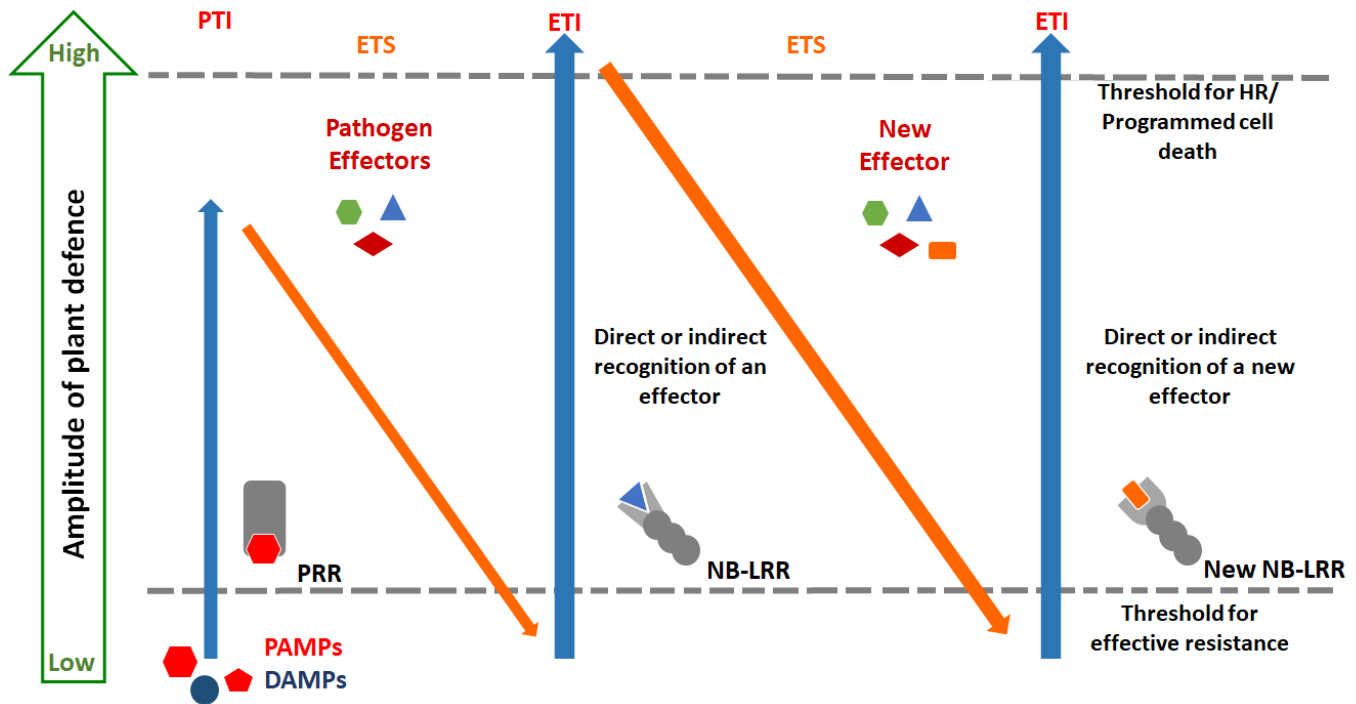


Figure 2.10: Schematic representation of innate immunity directed towards phytopathogens *via* the Zig-Zag model (adapted and compiled from Jones & Dangl, 2006; Zvereva & Pooggin, 2012). The amplitude of plant defence is proportional to $[PTI - ETS + ETI]$. Initially, phase 1 shows the detection of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) *via* pattern-recognition receptors (PRRs) to induce pattern-triggered immunity (PTI). In the next phase pathogens that successfully evaded PTI produce effectors to suppress PTI and further promote pathogen spread, leading to effector-triggered susceptibility (ETS). Phase 3 illustrates the recognition of one effector either directly or indirectly *via* an NB-LRR protein, resulting in effector-triggered immunity (ETI), a rapid amplified version of PTI that often passes the threshold resulting in a hypersensitive response (HR) and programmed cell death. Pathogen patterns become altered or modified (phase 4), resulting in new effectors that can suppress ETI. New NB-LRR proteins are then subsequently evolved to recognise the new effector leading to ETI again.

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2.6 References

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Chapter 3

Plant metabolomics for biomarker discovery: Key signatory metabolic profiles for the identification and discrimination of oat cultivars

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Abstract

Background: The first step of any crop introduction - or breeding program requires cultivar or variety identification and characterisation, and therefore rapid identification methods would greatly improve registration, breeding, seed, trade and inspection processes. Metabolomics, the youngest of the -omics trilogy, has proven to be indispensable in interrogating cellular biochemistry and phenotyping. Furthermore, metabolic fingerprints are chemical maps that can provide detailed insights into the molecular composition of a biological system under consideration. **Aim:** In this study, metabolomics was applied to unravel differential metabolic profiles of various oat (*Avena sativa*) cultivars (Magnifico, Dunnart, Pallinup, Overberg, and SWK001) and to identify signatory biomarkers for cultivar identification. **Methods:** The respective cultivars were grown under controlled conditions with 12 h light/ dark cycles in average temperatures ranging from 25-28 °C. Once the plants reached 3-week maturity (seedling stage), the leaves and roots were harvested for each cultivar. Metabolites were extracted using an 80% methanol extraction method. The extracts were analysed using an ultra-high-performance liquid chromatography (UHPLC) system coupled to a qTOF high definition mass spectrometer analytical platform. **Results:** The generated data were processed and analysed using multivariate statistical methods. Principal component analysis (PCA) models were computed for both leaf and root data. The PCA scores plots showed cultivar-related clustering of the samples, pointing to underlying differential metabolic profiles of these cultivars. Further multivariate analyses of the data were performed to profile differential signatory markers, which included carboxylic acids, amino acids, fatty acids, phenolic compounds (hydroxycinnamic acids and hydroxybenzoic acids and associated derivatives) and flavonoids among the respective cultivars. **Conclusion:** Based on the results, the cultivars were successfully distinguished from one another (in profiles derived from both leaves and roots) using the above mentioned key signatory metabolic markers. Thus the study demonstrates that metabolomics can be used as a rapid tool to differentiate cultivars.

3.1 Introduction

Food demand has been rapidly increasing with the overall growth in the world population, which is expected to reach around 9.7 billion by the year 2050 (Tilman *et al.*, 2011). Now more than ever, crop improvement and plant breeding studies have become imperative in ensuring

food security and sustainability (Hundleby and Harwood, 2019). The primary step involved in plant breeding, inspection, registration, trade and seed production requires the identification of cultivars and varieties, and therefore a rapid and effective method for cultivar fingerprinting is required (Korir *et al.*, 2013). Over the years, plant breeding has been greatly improved for unravelling the molecular basis of complex traits using genomic analyses and next-generation sequencing methods (Edwards *et al.*, 2013). Currently, plant breeding methods have integrated phenotypic traits with a range of marker-assisted selection techniques to more efficiently determine trait outcomes (Boopathi *et al.*, 2020). Although genetic markers have been at the forefront of plant breeding efforts, many limitations have restricted the use thereof in analysing complexities arising from genotype $\times$ environment interactions (environmental plasticity), polygenic inheritance and epistasis (which is referred to as the action of one gene on another) (Yandeau-Nelson *et al.*, 2015).

Metabolomics, a systems biology approach to interrogate cellular biochemistry and metabolism, offers unique possibilities that can be incorporated into unravelling these complexities to gain a genotype $\times$ metabolite $\times$ phenotype understanding that can be applied in plant breeding. The phenotype is an observable reflection that results from complex interactions between the genotype and the environment, with the former also able to prime multiple phenotypes (Gienapp *et al.*, 2017). These interactions can result in various success rates in reproduction and cause subsequent alterations in the genotype, as summarised in the abridged illustration (**Figure 3.1**). To bridge the gap between the genotype and phenotype, Handakumbura *et al.* (2019) proposed metabolomics as a means to provide insight into how genotypic variation affects phenotypic diversity in plants.

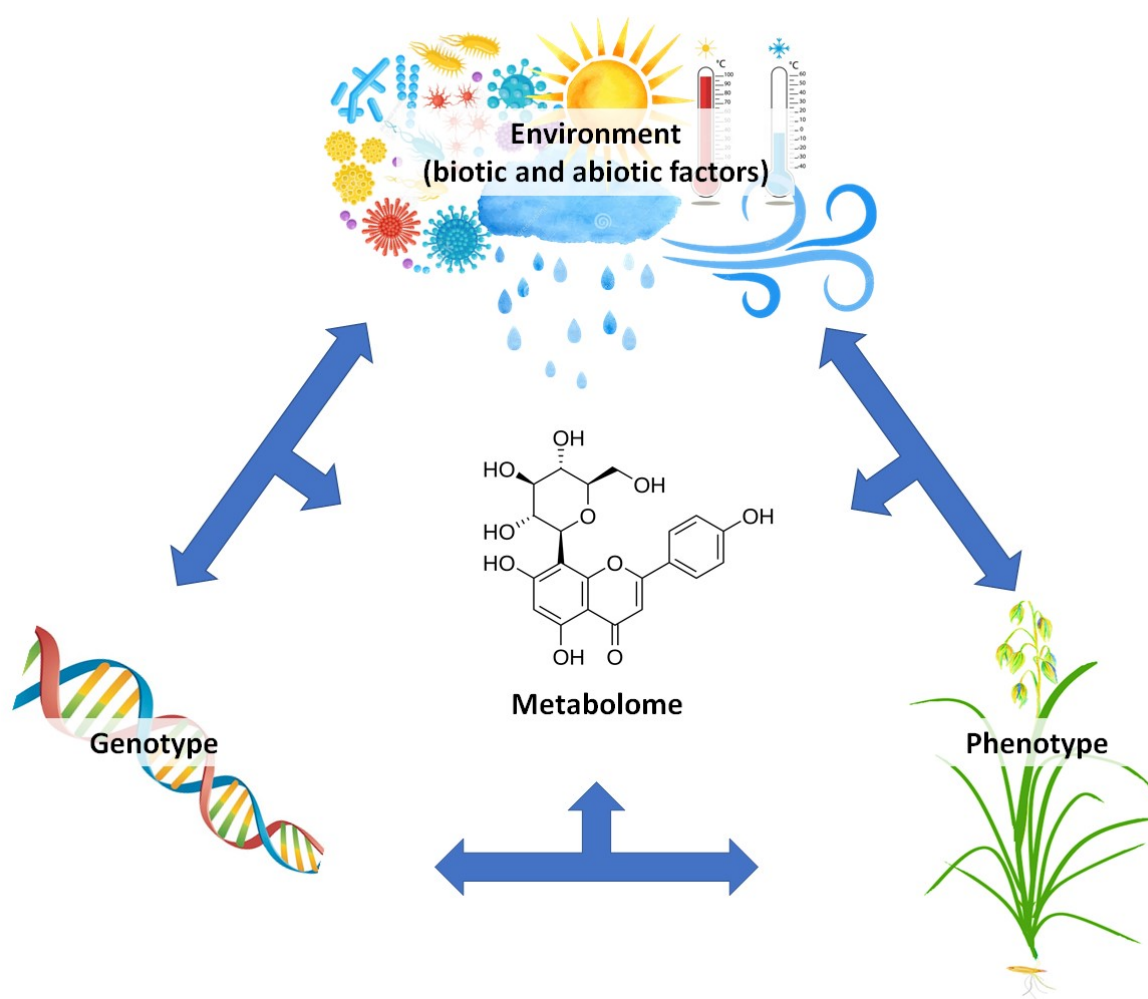


Figure 3.1: Triangular arrangement illustrating the genotype $\times$ environment $\times$ phenotype interactions, with the metabolome at the core, bridging the gap between the genotype and phenotype. The metabolome is the final recipient of biological information flow and carries imprints of genetic and environmental factors. It is more sensitive to perturbations in both metabolic fluxes and enzyme activity than either the transcriptome or proteome and is thus a reflection of the phenotype. Quantitative, global measurements of the metabolome, therefore, provide an exploration of cellular metabolism, revealing patterns and functional signatures of the biochemical landscape and cellular physiology of the system under consideration (Rosato *et al.*, 2018; Handakumbura *et al.*, 2019; Hamany-Djande *et al.*, 2020).

In the past, metabolomics has proven crucial for studying plant $\times$ environment interactions, (*e.g.* adaptive responses towards biotic and abiotic stresses), and has been applied in metabolomics-assisted breeding of crops. So far, great progress has been made in the development of metabolomics tools for crop improvement. However, there are still bottlenecks in metabolomics with regard to analytical technologies and tools used for data mining and interpretation. Some of these limit metabolome coverage, the maximization of metabolomics data and the annotation of extracted metabolites (Kumar *et al.*, 2017; Razzaq *et al.*, 2019; Ivanisevic & Want, 2019). The metabolites that have been discovered were divided into

different classes based on their structure, biochemical properties, and functions (see section 2.3). Metabolomics, defined as the comprehensive qualitative and quantitative analysis of all metabolites in a biological system, is an established omics technology that holds promise in agricultural research, therefore metabolomics has become an indispensable tool in various plant sciences studies (Tugizimana *et al.*, 2013; Kapoore & Vaidyanathan, 2016). In plants, metabolites are known to play important roles in crop yield, nutritional quality, growth and development, as well as in plant defence against environmental stresses (Hussein & El-Anssary, 2018; Isah, 2019). Different metabolomic applications have therefore been developed to elucidate plant responses and mechanisms under different conditions to determine metabolic profiles for use in crop improvement (Kumar *et al.*, 2017). As such, metabolomics allows the predictive discovery of biomarkers, independent of genetic and environmental variation. These metabolite biomarkers provide invaluable information on biochemical mechanisms that underly phenotypic traits and can be used in the development of targeted methods for breeding programs (Steinfath *et al.*, 2010; Peng *et al.*, 2015; Razzaq *et al.*, 2019). Plant metabolites are increasingly incorporated into breeding programs for the prediction of phenotypic traits and thus provides an early detection tool for identifying favourable traits. In this study, metabolomics tools and approaches were applied in order to develop a profiling methodology able to discriminate between various oat (*Avena sativa* L.) cultivars.

3.2 Materials and Methods

3.2.1 Plant cultivation

Seeds of five oat (*Avena sativa* L.) cultivars: **Magnifico**, **Dunnart**, **Pallinup**, **Overberg** (Agricol, Pretoria, South Africa), and **SWK001** (ARC Small Grain Institute, Bethlehem, South Africa), were obtained and cultivated in triplicate. All cultivars were grown in germination mixture (Culterra, Muldersdrift, South Africa) under greenhouse conditions: a light/dark cycle of 12 h/12 h, with a light intensity of about 84 $\mu\text{mol}/\text{m}^2/\text{s}$ and temperature between 25–28 °C. Once the plants reached 3-week maturity (seedling stage or three-leaf stage), the leaves and roots were harvested, frozen in liquid nitrogen to quench metabolic activity, and stored at –80 °C until metabolite extraction. The experimental design included three independent biological replicates and the experiments were repeated twice.

3.2.2 Metabolite extraction and sample preparation

Liquid nitrogen was added to the leaf and root material, which were then crushed into powder form using a mortar and pestle. One gram per sample was weighed into a clean falcon tube and 10 ml of 80% cold aqueous methanol (4 °C) was added (m/v ratio of 1:10). The methanol used was analytical grade (Rochelle Chemicals, Johannesburg, South Africa). The mixture was then homogenized using a probe sonicator (Bandelin Sonopuls, Berlin, Germany) set to 55% power for 10 s per sample. Equipment was cleaned between samples to prevent cross-contamination. The homogenates were centrifuged at $5100 \times g$ for 20 min at 4 °C in a benchtop centrifuge after which the supernatants were kept and concentrated by evaporating the methanol under vacuum to approximately 1 mL using a rotary evaporator set to 55 °C. The concentrated samples were transferred to 2 mL microcentrifuge tubes and dried in a centrifugal evaporator under vacuum. The dried extracts were then reconstituted by dissolving in 500 μ L of 50% aqueous methanol (LC-grade, Romil Pure Chemistry, Cambridge, UK). The samples were subsequently filtered through nylon syringe filters (0.22 μ m) into chromatography vials fitted with 500 μ L inserts, capped, and kept at 4 °C until analysis.

3.2.3 Ultra-high-performance liquid chromatography (UHPLC) analyses

An Acquity UHPLC system (Waters Corporation, Manchester, UK) was used to analyse 2 μ L of each sample, separated into its respective components using a binary solvent on an HSS T3 reverse-phase column (Waters Corporation, Billerica, MA, USA; $2.1 \times 150 \text{ mm} \times 1.7 \mu\text{m}$). The solvents used were MilliQ water (solvent A) and acetonitrile (solvent B) (Romil Chemistry, Cambridge, UK), both containing 0.1% formic acid (Sigma, Munich, Germany) and 2.5% isopropanol (IPA, Romil, Cambridge, UK). The run was set to 30 min per 2 μ L injection with an elution gradient carried out via a binary solvent system at a flow rate of 0.4 mL min^{-1} . The initial conditions were 95% A and 5% B and held for 1 min. A gradient was applied to change the chromatographic conditions to 10% A and 90% B at 25 min and changed to 5% A and 95% B at 25.10 min. These conditions were held for 2 min and then changed to the initial conditions at 28 min. The analytical column was allowed to calibrate for 2 min before the next injection. Pooled quality control (QC) samples were also prepared to condition the LC-MS system, and assess the reliability and reproducibility of each analysis (Broadhurst *et al.*, 2018).

Additionally, blank samples (50% MeOH) were also randomly included in the run to monitor the background noise. Each sample was analysed in triplicate (technical replicates), and together with the three biological replicates, this generated $n=9$ for each experiment, to account for analytical variability.

3.2.4 Quadrupole time-of-flight mass spectrometry (q-TOF-MS)

A high definition SYNAPT G1 Q-TOF mass spectrometry system, controlled by MassLynx XSTM software (Waters Corporation, Manchester, UK), was coupled to the chromatography system to detect metabolites and acquire data in both positive and negative electrospray ionisation (ESI) operation modes. A reference calibrant, leucine enkephalin (554.2615 Da) was set as the lockmass and allowed for typical mass accuracies from 1–3 mDa. The respective capillary and sampling cone voltages were set as 2.5 kV and 30 V. The desolvation temperature used was 450 °C, with the source temperature set to 120 °C, cone gas flow was set to 50 L/h, and the desolvation gas flow set to 550 L/h. An m/z range of 50–1200 Da was set with a scan time of 0.1 s. The desolvation-, collision- and cone gas used at a flow rate of 700 L/h was high-purity nitrogen. Data was acquired using five different collision energies (MS^E), ramping from 0–50 eV to cause fragmentation of the initial ions so as to ensure that as much information regarding the structures of the respective compounds could be obtained for downstream structural elucidation and metabolite annotation (Tugizimana *et al.*, 2014; Zeiss *et al.*, 2018).

3.2.5 Data analyses

The data sets obtained were explored and processed using MarkerLynx XSTM software (Waters Corporation, Manchester, UK). The software makes use of a patented algorithm called ApexTrack. The following parameters were used for processing: retention time (Rt) range 2–25 min and m/z range 150–1200 Da. The Rt window was set to 0.20 min and the mass window to 0.05 Da. The mass tolerance was 0.05 Da and the intensity threshold was set to 150 counts. The generated data matrices were exported into ‘soft independent modelling of class analogy’ (SIMCA) software, version 14 (Umetrics, Umea, Sweden) for multivariate data analysis (MVDA). Unsupervised models, namely principal component analysis (PCA) and hierarchical clustering analysis (HCA) were used to reduce the dimensionality of the data sets and to explore the underlying structures and characteristics of the data. Supervised orthogonal projection to

latent structures discriminant analysis (OPLS-DA) was used for binary classification analyses of cultivars, identifying thus discriminatory ions among the different cultivars. The OPLS-DA models were validated using rigorous methods (Trygg *et al.*, 2007; Tugizimana *et al.*, 2013; Worley & Powers, 2013). The roles of these MDVA tools in metabolomics workflow are further described in **section 3.3.2**.

3.2.6 Metabolite annotation and semi-quantitative comparisons

Metabolites were putatively identified based on their respective (measured) accurate masses (based-on which elemental compositions were computed using the MarkerLynx XS software tool), and fragmentation information (for structural elucidation). Each putatively suggested empirical formula was exported and searched for in various databases such as MetaCyc (<https://metacyc.org/>), plant metabolic network (PMN) (<https://plantcyc.org/>), ChemSpider, Mass bank of North America (<https://mona.fiehnlab.ucdavis.edu/>), Dictionary of Natural Products (www.dnp.chemnetbase.com) and the Kyoto Encyclopedia of Genes and Genomes (KEGG, www.genome.jp/kegg/). Processed data matrices were also exported from MarkerLynx XS software to the ‘Taverna workbench’ containing an in-house library and allowing a high throughput automated assignment of putative metabolite identities based on measured accurate masses and other collected spectral features (for a detailed description see Brown *et al.*, 2011). Metabolites were putatively identified to level 2 of the Metabolomics Standards Initiative (MSI) unless specified otherwise (Sumner *et al.*, 2007).

Furthermore, MetaboAnalyst 4.0 (Chong *et al.*, 2018) was utilised for additional integrative data analyses. Data pre-treatment (integrity, missing values, filtering and normalisation) was performed prior to downstream chemometric and statistical modelling. A comparison of the magnitude and presence of the identified metabolites among the various cultivars was performed *via* heatmap analyses using a Pearson distance measure and Ward clustering algorithm (Xia *et al.*, 2009; Chong *et al.*, 2018). Partial least square-discriminant analysis (PLS-DA) was also used to mine the data *via* MetaboAnalyst for the comparison and visualisation of the relative abundances of the identified metabolites across the various cultivars. VIP scores plots, derived from the generated PLS-DA, were generated to indicate the key discriminatory metabolites with VIP scores of >0.5 which are considered significant in discriminating between the cultivars. Additionally, to further visualise changes among the

discriminatory metabolites across the various cultivars, radar plots were constructed based on the averages of the relative intensities and illustrated as log-transformed values (**section 3.3.3**).

3.3 Results and Discussion

3.3.1 Differential chromatographic-mass spectrometric analyses of respective oat cultivars

When compared to other cereal crops, oat has been greatly underrated, despite containing a range of unique compounds and nutrients that are greatly beneficial for human health and reduces incidences of certain degenerative diseases (Stewart & McDougall, 2014). Oat is also considered superior due to its hardiness as well as the ability to thrive and withstand environmentally poor conditions where other cereals seem to be lacking (Saini *et al.*, 2019). These benefits are greatly attributed to the rich diversity of secondary metabolites that oat contains such as phenolic acids, flavonoids, phytosterols, carotenoids, avenanthramides, avenacosides and avenacins (Belobrajdic & Bird, 2013; Sang & Chu, 2017). In this study, five oat cultivars were analysed to characterize their metabolic profiles, where methanolic extracts of leaf and root tissues of the respective cultivars were separated (and detected in both positive and negative ESI modes) on a UHPLC-QTOF-MS analytical system. Initial optimisation studies indicated that most extractable metabolites ionised better in the ESI (-) mode; accordingly only these data sets are further presented and illustrated. The base peak intensity (BPI) chromatograms (**Figure 3.2**) provide a visual presentation/description of the similarities and differences between the respective cultivars and illustrate the complexity of their metabolic profiles. The coupling of chromatography to mass spectrometry provided a highly sensitive analytical platform that allowed for the simultaneous detection of a range of metabolites to provide a more holistic insight into the metabolic composition of the biological samples. Although the LC-MS system is extremely useful in the separation and accurate mass determination of the respective constituents, biologically useful information is further obtained from chemometric analyses.

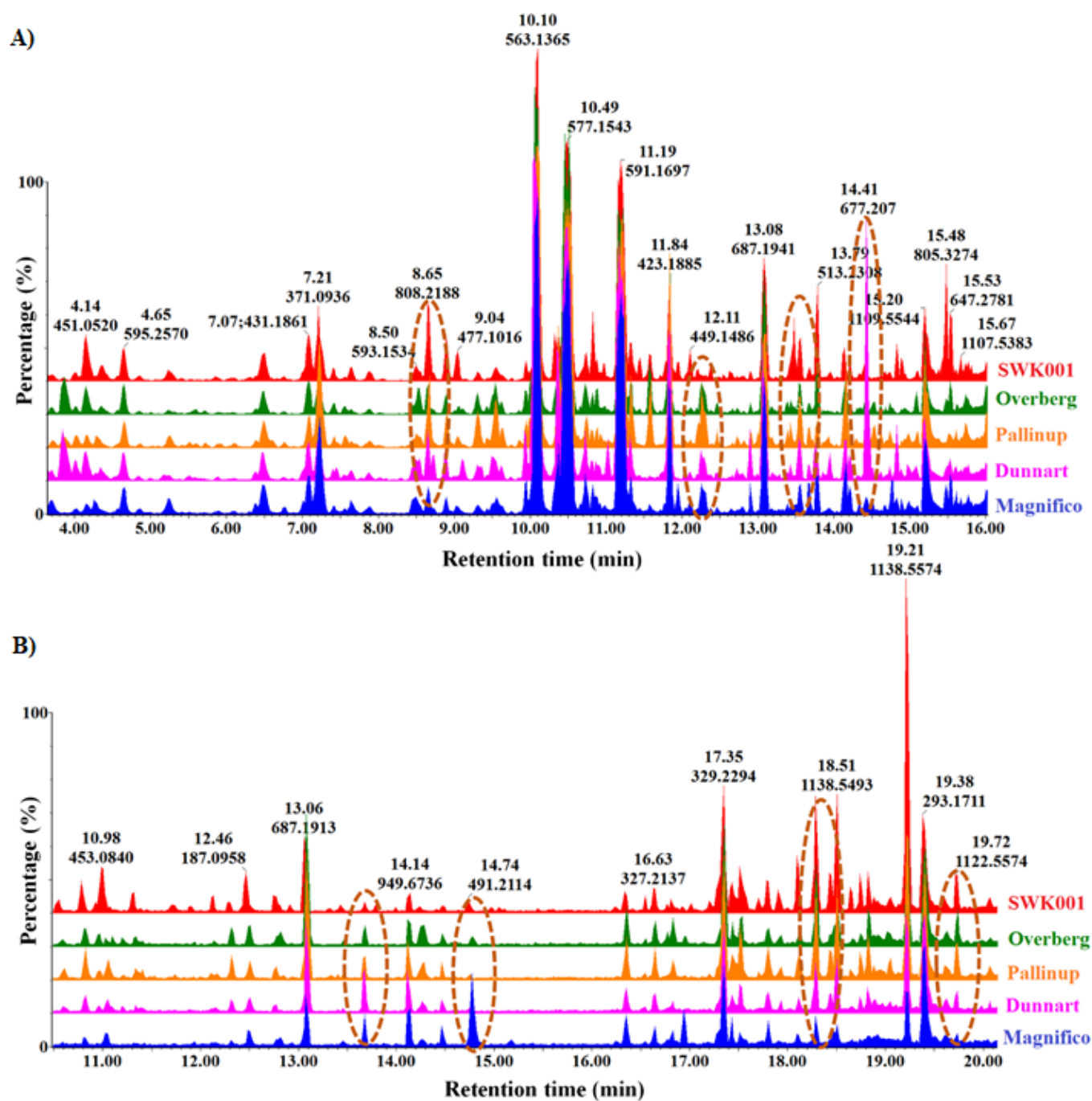


Figure 3.2: Ultra-high-performance liquid chromatography (UHPLC) coupled to mass spectrometric (MS) detection. The figure compares base peak intensity (BPI) MS chromatograms of methanol (A) leaf and (B) root extracts from five oat cultivars (SWK001, Overberg, Pallinup, Dunnart & Magnifico) at the seedling stage. These represent the chromatographic separation based on the polarity of the different compounds in negative ionisation mode (ESI⁻), separated on an HSS T3 reverse-phase column. The dashed oval structures point out some cultivar-exclusive variations that illustrate differences in the phytochemical profiles of the cultivars.

3.3.2 Chemometric analyses for profiling the measured oat metabolomes

Due to the complexity and multi-dimensionality of metabolomic data, appropriate statistical and chemometric tools are required to obtain chemical information and convert it into biological knowledge (Boccard & Rudaz, 2014). Chemometrics is the science of extracting useful information from complex data sets through pattern recognition and machine learning algorithms (Brereton, 2015; Tebani *et al.*, 2018). PCA is a multivariate technique that increases the interpretability and minimises the loss of biological information by reducing the dimensionality of complex datasets (Jolliffe & Cadima, 2016). The underlying structures and characteristics of the data are thus revealed by this unsupervised, explorative method. The illustrated PCA models (**Figure 3.3**) show distinct clustering of the five respective cultivars (**Magnifico**, **Dunnart**, **Pallinup**, **Overberg**, and **SWK001**), which points to underlying differential metabolic profiles from the leaf (**Figure 3.3A**) and root (**Figure 3.3B**) tissues. The model illustrates both similarities and differences within (PC2/3) and between (PC1) the cultivar groupings. This PCA-revealed differential clustering correlates to the differences previously visualised by the chromatographic separation (**Figure 3.2**).

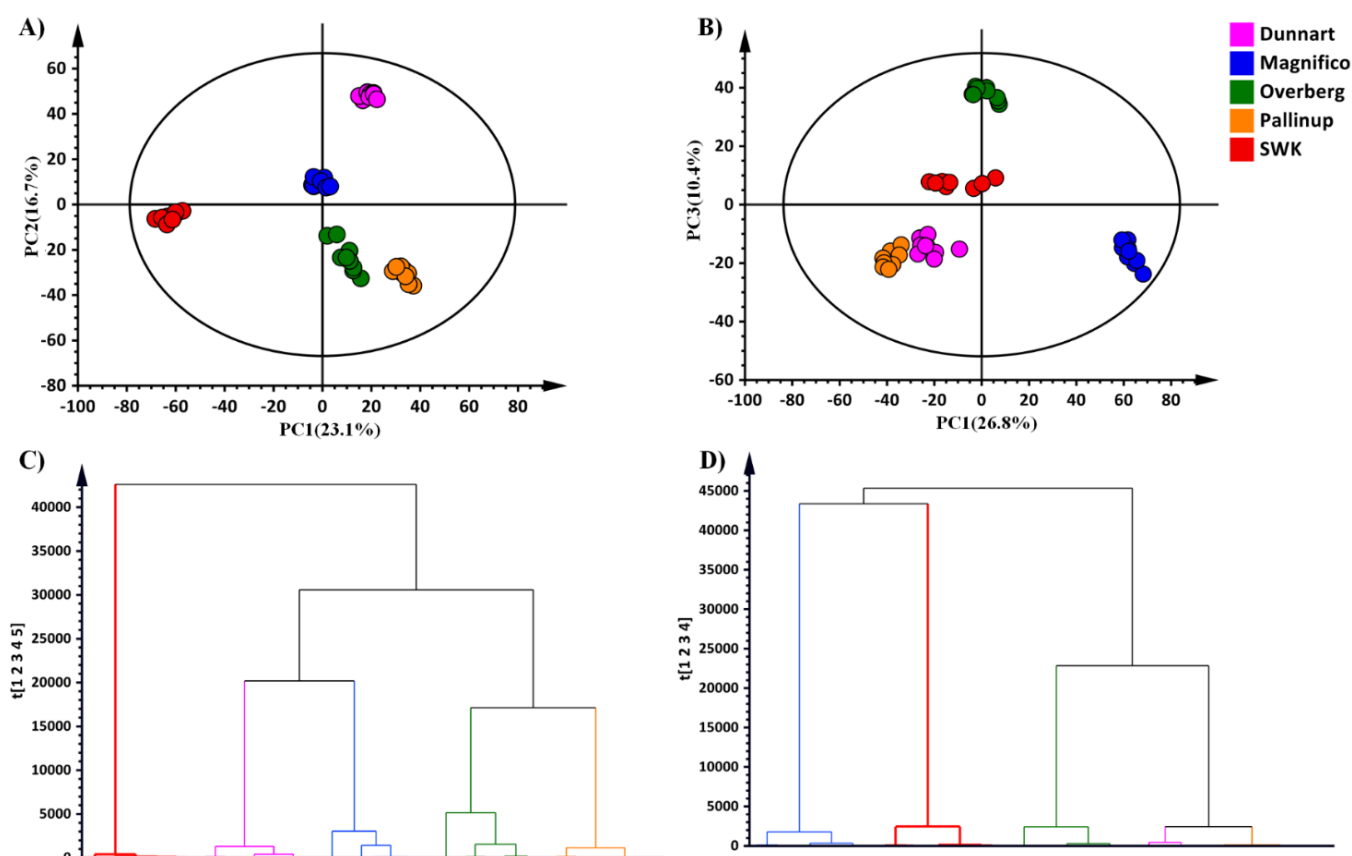


Figure 3.3: Principal component analysis (PCA) of five oat cultivars with the corresponding hierarchical cluster analysis (HCA) dendrogram. PCA scores plots indicate the clustering and general grouping among the five cultivars (**Overberg**, **Pallinup**, **Dunnart**, **Magnifico** & **SWK001**) extracted from (A) leaf and (B) root tissues, analysed in ESI- mode. The HCA dendrogram shows the hierarchical structure of the data indicating that (C) **Pallinup** is phytochemically more similar to **Overberg** and **Dunnart** to **Magnifico**. In comparison, **SWK001** is the most different from the other cultivars concerning their leaf metabolic profiles. (D) HCA dendrogram illustrating the root metabolic profiles; **Pallinup** and **Dunnart** are similar, and cluster closely with **Overberg**. In comparison, **Magnifico** and **SWK001** are metabolically different from the other three cultivars based on the extracted profiles.

In addition to PCA modelling, another unsupervised technique, namely HCA, is often used to cluster high-dimensional data into a dendrogram based on dissimilarity and similarity of the samples. In a bottom-up representation (Figure 3.3) the algorithm clusters each observation based on their differences and further proceeds by joining the most similar clusters at each step in an iterative manner. The resulting dendrogram illustrates that the metabolic profiles of the leaf tissues (Figure 3.3C) of **Magnifico** and **Dunnart** appear to be closely related; similarly in the case of **Pallinup** and **Overberg**, with **SWK001** appearing to be most metabolically different and clustering at the far left (Handorf *et al.*, 2018). The cultivars also cluster separately based on their profiles extracted from root tissues (Figure 3.3D); in this case, however, **Dunnart** and **Pallinup** seem to be most similar metabolically, and in turn, are grouped with **Overberg**. **Magnifico** and **SWK001** in this case are the most metabolically different from the other cultivars and similar to each other. It is of interest that the unsupervised, explorative method

not only underscored differences between cultivars but also highlighted differences between the extracts from roots and leaves of these cultivars. The source-to-sink model describes differences between various plant tissues based on their environment as well as the synthesis and transport of various nutrients required for growth and development (Lemoine *et al.*, 2013). Source tissues are often described as net exporters of resources required for plant growth, like carbon or nitrogen, while sink tissues are net importers responsible for resource absorption. Mature leaves are net sources of carbon but sink for nitrogen, while root tissues are net sources of nitrogen but sink for carbon (White *et al.*, 2016). Another example contributing to the differences among the respective tissues is the presence of secondary metabolites. These may be uniquely synthesised by either the leaves or roots, such as the case for avenacins, which are triterpenoid saponins found in roots whilst the leaves contain steroidal saponins known as avenacosides. Both compounds serve a similar purpose but are respectively confined to the various plant tissues (Mugford & Osbourn, 2012). To obtain more in-depth information regarding the differential metabolic profiles revealed by PCA modelling, supervised methods such as orthogonal projections to latent structures discriminant analysis (OPLS-DA) were used.

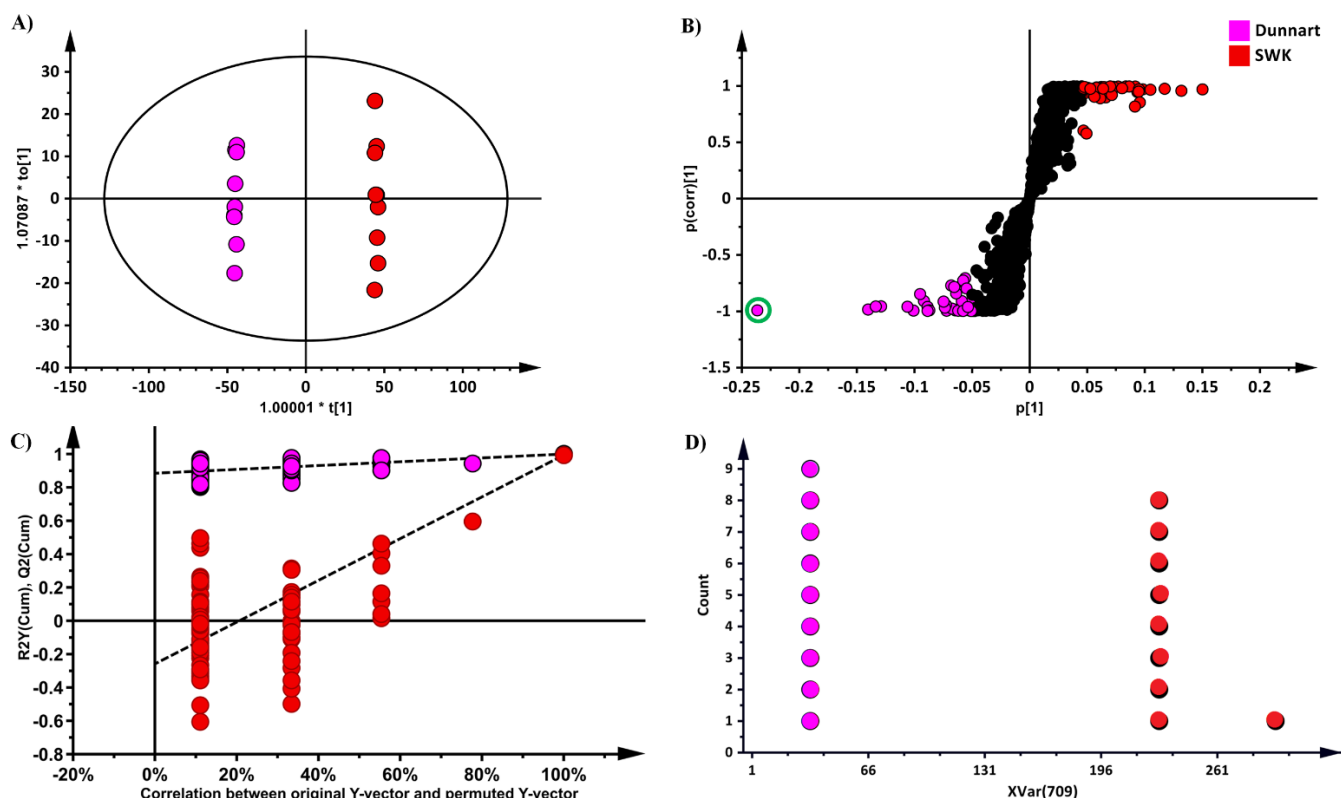


Figure 3.4: An orthogonal projection to latent structures discriminant analysis (OPLS-DA) model of two representative cultivars: Dunnart and SWK001. (A) An OPLS-DA scores plot summarising the relationship among different datasets to visualise group clustering between the Dunnart and SWK001 cultivars based on their leaf-extracted metabolic profiles obtained in ESI(-) MS mode ($R^2=0.998$, $Q^2=0.971$, CV-ANOVA p -value= 5.8941×10^{-14}). (B) The corresponding OPLS-DA loadings S-plot. The pink and red circles indicate the values situated far out ($p[1] > 0.05$, < -0.05) in the S-plot, indicating statistically significant ions that are possible discriminatory variables between the Dunnart and SWK001 cultivars respectively. (C) Permutation test plot ($n = 100$) for the OPLS-DA model (A) was used to validate the predictive capability. (D) Dot plot illustrating strong discrimination between the cultivars for the selected variable (circled in green on the S-plot) as there is no overlap between the groups.

OPLS-DA modelling showed sample classification in the scores-space between different experimental groups, as depicted in **Figure 3.4A**. With the SWK001 and Dunnart cultivars, clear clustering and group separation are shown. As a supervised method, OPLS-DA is often considered a model that is prone to overfitting data, therefore rigorous model validation methods are used to ensure validity and reliability of the computed model (Worley & Powers, 2016). The reliability of the models was tested using cross-validation analysis of variance (CV-ANOVA) where the significant models had p -values of <0.05 . Furthermore, the performance of the OPLS-DA models was evaluated using receiver operating models (ROC) where perfect classification was depicted as the ROC curve passed through the top left corner, indicating perfect sensitivity and specificity (**Supplementary Figure S3.1**). Finally, permutation tests were performed where the OPLS-DA models were statistically shown to be better than the generated permutation models with an R^2 and Q^2 being higher for the OPLS-DA model (**Figure**

3.4C). The loadings S-plots (**Figure 3.4B**) were used to target and select statistically significant discriminatory ions among the different cultivars. OPLS-DA models and their corresponding loadings S-plots were constructed for all cultivars in both leaf and root tissues (20 in total for each tissue type – and model infographics are available on request). Furthermore, each selected variable from the S-plot was evaluated using a dot plot (**Figure 3.4D**) that computes each observation as a unit and subsequently sorts each component into “bins” that represent sub-ranges. Strong discriminating variables show no overlap between the groups as can be seen in **Figure 3.4D**.

3.3.3 Differential metabolic profiles based on discriminatory ions

Following the selection of discriminant ions from the respective loadings S-plots, a list of putatively identified metabolites was compiled and is presented in **Table 3.1**. The statistically significant variables were annotated as described in experimental **section 3.2.6**. The possible chemical structures were then explored by further inspection of the generated fragmentation patterns under various collision energies MS^E (**Figure 3.5**). The annotated metabolites thus represent the discriminatory compounds that allowed for differentiation among the different cultivars. These compounds were placed in the following metabolite classes: carboxylic acids, amino acids, fatty acids, phenolics and flavonoids. In addition, a steroidal saponin (avenacoside A) was annotated in extracts from leaves and a triterpenoid saponin (avenacin A-1) in extracts of roots.

Among the groups of metabolites identified, carboxylic acids, also sometimes referred to as organic acids, can be described as compounds that contain a carboxyl functional group in their structure. These compounds are widely distributed in nature and involved in primary metabolism, responsible for growth and development (Badea & Radu, 2018). Citric acid was identified as a discriminatory ion in root extracts among the different cultivars. It forms part of the tricarboxylic acid (TCA) cycle and is a pivotal part of energy synthesis, and provides precursors for the biosynthesis of a range of secondary metabolites and amino acids in plants (Zhang & Fernie, 2018).

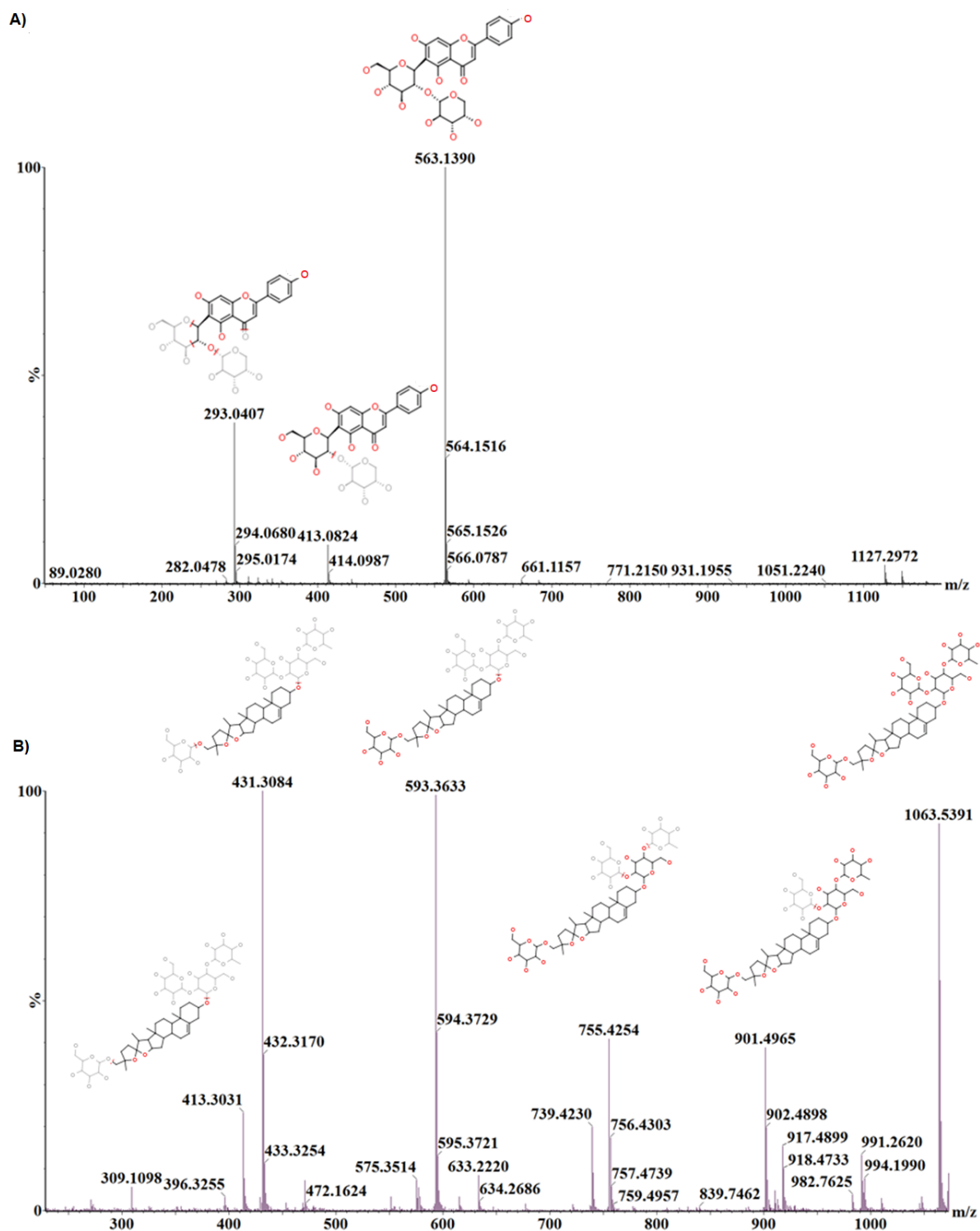


Figure 3.5: Representation of the use of fragmentation patterns for the annotation of secondary metabolites
(A) Isoviteixin 2''-O-arabinoside showing the parent ion at m/z 563 and two diagnostic fragment ions at m/z 413 and 293. **(B)** Avenacoside A at an m/z of 1063 showing diagnostic fragments (m/z 901, 755, 593, 431 and 413) with their structural changes that aid in the structural identification of the metabolite. The fragmentation spectrum enables confirmation of the elemental composition and provides useful hints to elucidate possible structural information by evaluating fragmentation patterns yielded at different collision energies MS^E.

Common substrates for the synthesis of amino acids include not only intermediates from the TCA cycle but also glycolysis and the pentose phosphate pathway. The latter primarily produces intermediates involved in the synthesis of phenylalanine, tyrosine and tryptophan (Tzin & Galili, 2010). Among these, phenylalanine and tryptophan presented as discriminatory metabolites in the leaves and roots. Tryptophan is involved in two distinct pathways to produce secondary metabolites. One such pathway starts with the decarboxylation of tryptophan by tryptophan decarboxylase (TDC) to initiate the synthesis of indole alkaloids. The tryptophan pathway also branches from the shikimate pathway at chorismate, where it is initially synthesised from anthranilate (another discriminatory metabolite) by anthranilate synthase. The secondary metabolites produced *via* these pathways have been known to play pivotal roles in the defence systems in various members of the grass family (*Poaceae*) (Ishihara *et al.*, 2007; Kokubo *et al.*, 2017). Anthranilate also plays an important role in the synthesis of avenanthramides (Av) which are phytoalexins that are produced in response to pathogen infection. Av has been found to form dimers and is incorporated into plant cell walls for reinforcement, thus they function in both the chemical and physical defence of oat against pathogens (Okazaki *et al.*, 2004; Li *et al.*, 2019).

Flavonoids are synthesised through the phenylpropanoid pathway (**Figure 3.9A**), where 4-coumaroyl-CoA is formed from cinnamic acid, which finally enters the flavonoid (**Figure 3.9B**) biosynthesis pathway (Falcone-Ferreira *et al.*, 2012). The first enzyme specific for the flavonoid pathway, chalcone synthase, produces chalcone scaffolds from which all flavonoids derive. Flavonoids most common in oat include apigenin, luteolin, tricetin, kaempferol, quercetin and their glycoside derivatives (Peterson, 2001; Krošlák *et al.*, 2016). The majority of metabolites identified were classified as flavonoids, with most being glycoside derivatives of apigenin, quercetin, kaempferol and tricetin. As described in **section 2.3.3**, flavonoids have a range of biological activities in plants such as antioxidant -, antimicrobial -, signalling -, allelopathic - and defence against environmental stressors (Panche *et al.*, 2016). Phenolic acids, as previously described, are synthesised *via* the phenylpropanoid pathway from phenylalanine through a process that commonly involves deamination, hydroxylation and methylation (Vogt, 2010).

Structurally, all phenolic acids are hydroxylated derivatives of cinnamic acid or benzoic acid. Hydroxycinnamic acid (HCA) derivatives commonly include ferulic acid, caffeic acid, sinapic acid and coumaric acid. Correspondingly, hydroxybenzoic acids include derivatives known as protocatechuic acid, gallic acid, vanillic acid and sinapinic acid (Lafay & Gil-Izquierdo, 2008). HCAs were abundantly identified among the various cultivars, with derivatives from coumaric acids, ferulic acid and sinapic acid commonly present. These phenolics are generally known to be significant in plant development, particularly in lignin and pigment biosynthesis, and provide structural and scaffolding support to plants (Bhattacharya *et al.*, 2010).

Plants also produce a range of fatty acids, some of which are presented as discriminatory among the cultivars. Commonly plants produce palmitic -, oleic -, linoleic - and linolenic acids, and in this study, oleic acid derivatives were frequently identified among the cultivars. Oleic acid is converted to linoleic acid which, in turn, is converted to linolenic acid (Aid, 2019). Oleic and linoleic acids are known to constitute the two major unsaturated fatty acids in plants and are involved in a range of biological activities, some of which include antifungal properties, and also in the synthesis of important defence signalling molecules such as jasmonates (Walley *et al.*, 2013; He *et al.*, 2020).

Two saponin molecules were also identified in the leaves (avenacoside A) and the roots (avenacin A-1) of the respective cultivars. Avenacosides are biologically inactive phytoanticipins that are converted into biologically active 26-desglucoavenacosides by an enzyme known as avenacosidase in response to tissue damage or pathogen attack (Pecio *et al.*, 2013). The major mechanism of activity against pathogens is due to their ability to complex with sterols in the pathogen membrane and cause disruption in the membrane integrity. This process is thought to result in the formation of transmembrane pores by aggregation of the saponin with the sterol groups. The remaining sugar moieties of the active molecules have also been known to play an essential role in membrane-permeabilization and therefore, the removal of these sugar residues could result in loss of biological activity (Morrissey *et al.*, 2000; Du Fall & Solomon, 2011; Pecio *et al.*, 2013; Moses *et al.*, 2014). Avenacins have a similar mechanism of action against pathogens that attack the roots, however, they are already present in biologically active forms. Ultimately, these saponins are responsible for defence against pathogens via the formation of micelle-like aggregations between the saponins and sterols in the membrane (Armah *et al.*, 1999; Owatworakit *et al.*, 2013).

Data visualisation tools were used to illustrate the magnitude and presence of the respective metabolites in various cultivars with heatmap analysis (**Figure 3.5 & 3.6**). Here, the average integrated peak areas of the respective metabolites were used to construct heatmaps using statistical analysis software available on MetaboAnalyst (Chong *et al.*, 2018). The infographics show clear differences among the cultivars with respect to their various metabolic profiles. These profiles could prove useful in not only discriminating among the various cultivars but also providing useful information on possible resistance or susceptibility capabilities between them. Among the identified metabolites (**Table 3.1**), the differential metabolic profiles based on discriminatory ions present in the hydromethanolic extracts of the various cultivars were as follows: **Magnifico** contained 9 flavonoids, 5 phenols and avenacoside A in the leaves, and 3 amino acids, 1 carboxylic acid, 3 fatty acids, 2 flavonoids and 1 phenol in the roots. **Dunnart**, on the other hand, had 7 flavonoids, 4 phenols and avenacoside A in the leaves, and 1 amino acid derivative, 1 carboxylic acid, 2 fatty acids, 2 flavonoids, 1 phenol and avenacin A-1 in the roots. **Pallinup** showed a metabolic profile containing 3 fatty acids, 9 flavonoids and 6 phenols in the leaves, and 1 amino acid, 1 fatty acid, 1 flavonoid, 1 phenol and avenacin A-1 in the roots. **Overberg** had 1 amino acid, 10 flavonoids, 4 phenols and avenacoside A in the leaves, and 3 amino acids, 1 carboxylic acid, 3 fatty acids, 2 flavonoids and 5 phenols in the roots. Lastly, **SWK001** showed a metabolic profile containing 1 amino acid, 2 fatty acids, 5 flavonoids, 5 phenols and avenacoside A in the leaves, and 3 amino acids, 1 carboxylic acid, 1 fatty acid, 1 flavonoid, 4 phenols and avenacin A-1 in the roots. Based on these differential metabolic profiles clear overlap and differences can be seen among the cultivars.

Table 3.1 List of key signatory metabolites extracted and putatively identified from leaves and roots of the oat cultivars **Magnifico (Mag)**, **Dunnart (Dun)**, **Pallinup (Pal)**, **Overberg (Over)** & **SWK001 (SWK)**. These discriminating metabolites were identified based on OPLS-DA S-plots, with a rigorous statistical validation (as explained in the text – **Figure 3.4**). These reported metabolites had VIP scores > 1.0.

Putative Identification	Molecular Formula	ESI	m/z	Rt (min)	Metabolite Class	Cultivar				
						Mag	Dun	Pal	Over	SWK
Leaves										
Coumaric acid	C ₉ H ₈ O ₃	Neg	163.0379	3.35	Phenolic acid			*		*
Tryptophan	C ₁₁ H ₁₂ N ₂ O ₂	Neg	203.081	2.48	Amino acid				*	*
Hydroxyoctadecatrienoic acid	C ₁₈ H ₃₀ O ₃	Neg	293.208	21.49	Fatty acid			*		
Dihydroxybenzoic acid glucoside	C ₁₃ H ₁₆ O ₉	Neg	315.0742	2.52	Phenolic acid	*				*
Glabranin	C ₂₀ H ₂₀ O ₄	Neg	323.1326	2.94	Flavonoid	*			*	
Trihydroxyoctadecadienoic acid	C ₁₈ H ₃₂ O ₅	Neg	327.2153	16.63	Fatty acid			*		*
Trihydroxyoctadecenoic acid	C ₁₈ H ₃₄ O ₅	Neg	329.23	17.34	Fatty acid			*		*
Caffeoylshikimic acid	C ₁₆ H ₁₆ O ₈	Neg	335.0422	2.24	Phenolic acid	*				*
Coumaroylquinic acid	C ₁₆ H ₁₈ O ₈	Neg	337.092	3.19	Phenolic acid		*		*	
Sinapoylglutamine	C ₁₆ H ₂₀ N ₂ O ₇	Neg	351.1257	6.48	Phenolic acid	*				
Feruloylquinic acid	C ₁₇ H ₂₀ O ₉	Neg	367.1008	4.01	Phenolic acid		*			
Sinapaldehyde glucoside	C ₁₇ H ₂₂ O ₉	Neg	369.1184	13.54	Phenolic acid		*	*	*	*
Dihydroferulic acid glucuronide	C ₁₆ H ₂₀ O ₁₀	Neg	371.0958	7.21	Phenolic acid	*		*		*
Syringin	C ₁₇ H ₂₄ O ₉	Neg	371.1346	16.0	Phenolic acid	*		*	*	
Sinapic acid glucoside	C ₁₇ H ₂₂ O ₁₀	Neg	385.1146	3.4	Phenolic acid			*		
Auriculoside	C ₂₂ H ₂₆ O ₁₀	Neg	393.1752	12.1	Flavonoid	*		*		*
Nobiletin	C ₂₁ H ₂₂ O ₈	Pos	403.1454	9.53	Flavonoid			*	*	
Sophoraflavanone G	C ₂₅ H ₂₈ O ₆	Neg	423.1856	11.83	Flavonoid	*		*	*	

Licoricidin	C ₂₆ H ₃₂ O ₅	Neg	423.2204	14.81	Flavonoid	*	*			*
Isovolubilin	C ₂₃ H ₂₄ O ₉	Neg	443.1328	16.81	Flavonoid			*	*	*
Isoquercetin	C ₂₁ H ₂₀ O ₁₂	Neg	463.0895	6.59	Flavonoid			*		
Xeractinol	C ₂₁ H ₂₂ O ₁₂	Neg	465.1028	12.97	Flavonoid				*	
Isorhamnetin 7-glucoside	C ₂₂ H ₂₂ O ₁₂	Neg	477.1038	9.03	Flavonoid	*		*		*
Oxalate derivative	C ₂₅ H ₂₄ O ₁₀	Neg	483.1281	12.2	Phenolic acid			*		
Isovitexin 2"-O-arabinoside	C ₂₆ H ₂₈ O ₁₄	Pos	563.1393	10.08	Flavonoid				*	*
Vitexin 2"-O-rhamnoside	C ₂₇ H ₃₀ O ₁₄	Neg	577.1545	10.49	Flavonoid	*	*		*	
Neocarlinoside	C ₂₆ H ₂₈ O ₁₅	Neg	579.1349	8.53	Flavonoid				*	
Acacetin-7-O-rutinoside	C ₂₈ H ₃₂ O ₁₄	Pos	593.186	11.2	Flavonoid	*	*		*	
Isovitexin 2"-O-glucoside	C ₂₇ H ₃₀ O ₁₅	Neg	593.1488	9.94	Flavonoid	*	*	*	*	
Isovitexin-7-O-glucopyranoside	C ₂₇ H ₃₀ O ₁₅	Pos	595.1499	8.5	Flavonoid		*	*		
Prenylkaempferol diglucoside	C ₃₂ H ₃₈ O ₁₆	Neg	677.207	14.41	Flavonoid	*	*			
Tricin ether glucopyranoside	C ₃₃ H ₃₆ O ₁₆	Pos	689.194	13.51	Flavonoid			*	*	*
Avenacoside A **	C ₅₁ H ₈₂ O ₂₃	Pos	1063.539	16.58	Steroidal saponin	*	*		*	*
Roots										
Pyroglutamic acid	C ₅ H ₇ NO ₃	Neg	128.033	1.16	Amino acid derivative	*	*		*	
Phenylalanine	C ₉ H ₁₁ NO ₂	Neg	164.068	1.67	Amino acid	*			*	*
Citric acid	C ₆ H ₈ O ₇	Neg	191.0163	1.16	Carboxylic acid	*	*		*	*
Tryptophan	C ₁₁ H ₁₂ N ₂ O ₂	Neg	203.081	2.49	Amino acid	*		*	*	*
Anthranilic acid dimer	C ₁₄ H ₁₂ N ₂ O ₄	Neg	271.07	2.49	Phenolic acid			*	*	
Kaempferol	C ₁₅ H ₁₀ O ₆	Neg	285.039	12.8	Flavonoid	*			*	*

Hydroxylinoleic acid	C ₁₈ H ₃₂ O ₃	Neg	295.15	23.87	Fatty acid	*			*	*
Octadecenedioic acid	C ₁₈ H ₃₂ O ₄	Neg	311.165	22.78	Fatty acid	*	*		*	
Dihydroxybenzoic acid glucoside	C ₁₃ H ₁₆ O ₉	Neg	315.069	1.67	Phenolic acid				*	*
Hydroxycoumarin glucoside	C ₁₅ H ₁₆ O ₈	Neg	323.097	1.68	Phenolic acid	*			*	*
Trihydroxyoctadecadienoic acid	C ₁₈ H ₃₂ O ₅	Neg	327.214	16.63	Fatty acid			*		
Trihydroxyoctadecenoic acid	C ₁₈ H ₃₄ O ₅	Neg	329.23	17.36	Fatty acid	*	*		*	
Feruloylquinic acid	C ₁₇ H ₁₉ O ₉	Neg	367.101	4.02	Phenolic acid				*	*
Sophoraflavanone G	C ₂₅ H ₂₈ O ₆	Neg	423.186	7.65	Flavonoid		*			
di-Sinapoylglucoside	C ₂₈ H ₃₂ O ₁₄	Neg	591.1693	11.2	Phenolic acid		*		*	
Tricin ether glucopyranoside	C ₃₃ H ₃₆ O ₁₆	Neg	687.192	13.06	Flavonoid	*	*	*	*	
Avenacin A-1	C ₅₅ H ₈₃ NO ₂₁	Neg	1092.55	18.49	Triterpenoid saponin		*	*		*

(*) Indicates metabolites that are presented as discriminatory ions in the respective cultivars.

(**) Metabolite identity was confirmed with an authentic analytical standard – MSI level 2.

Differences with regard to their relative intensities were also explored among the cultivars using heatmaps (**Figure 3.6 & 3.7**), colour-coded PCA scores plots (**Supplementary Figure S3.2**), pie charts (**Figure 3.9**) and radar charts (**Figure 3.10**). Among the cultivars, **Pallinup** had a number of metabolites that uniquely presented as discriminatory for this cultivar (hydroxyoctadecatrienoic acid, sinapic acid glucoside, oxalate derivative and isoquercetin in leaves, and trihydroxyoctadecadienoic acid in roots), although this does not necessarily always indicate absence in the other cultivars but only a greater relative intensity as can be seen in the generated heatmap (**Figure 3.6**). **Overberg** had 2 uncommon flavonoids that presented as discriminatory for this cultivar; xeractinol (a flavanol C-glucoside) and neocarlinoside (a tetrahydroxyflavone C-glycoside), as can be seen by the illustrated heatmap (**Figure 3.6**). The radar chart (**Figure 3.10A**) reiterates neocarlinoside as discriminatory in **Overberg** based on its intensity. Caffeoylshikimic acid is another example that was detected as a discriminatory metabolite for **Magnifico** and **SWK001**. **Figure 3.6** heatmap illustrates the presence of caffeoylshikimic acid among these cultivars and demonstrates the abundance to be greater in the **SWK001** cultivar compared to all the other cultivars, followed by **Magnifico**. This information is further confirmed by **Figure 3.9A** (pie chart), illustrating the pathways in which this metabolite is involved and how it is distributed amongst the cultivars, **Figure 3.10B** (radar chart) and by **Figure S2** (colour-coded PCA scores plot). **Dunnart** showed a greater abundance of feruloylquinic acid in both leaf and root tissue (**Figure 3.6 & Figure 3.7**), thus making this compound a discriminatory ion for this cultivar. Based on these examples it is clear that this method of cultivar profiling is sensitive enough to detect the presence of specific secondary metabolites among the different cultivars and generate relative intensity values, thus making it useful in cultivar profiling and comparison.

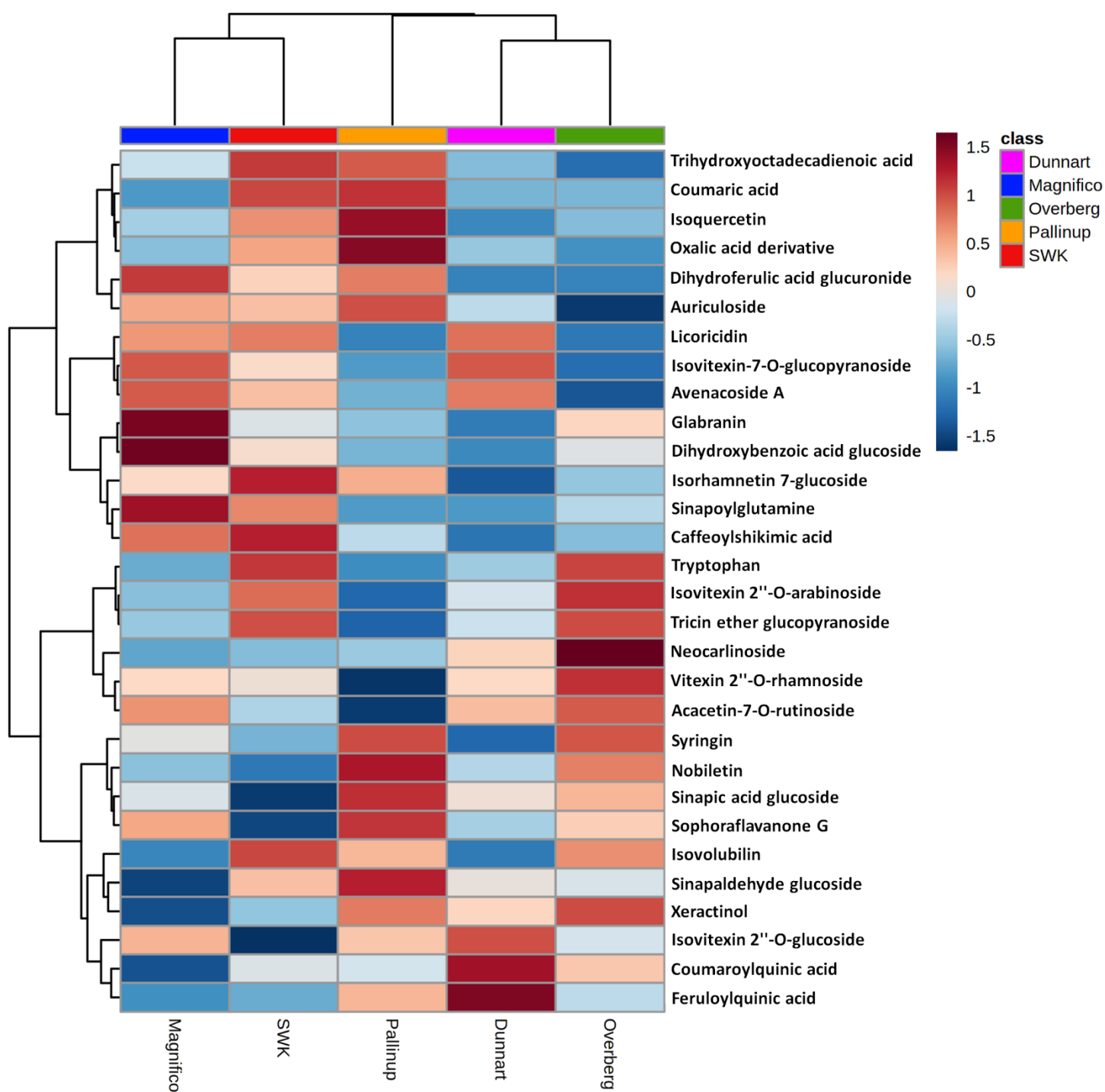


Figure 3.6: Individual peak intensities of putatively identified metabolites from oat leaves using heatmap analysis (Pearson distance and Ward's linkage rule) of the five cultivars **Overberg, **Pallinup**, **Dunnart**, **Magnifico** & **SWK001**.** The mean peak intensities of each annotated metabolite are shown after Pareto-scaling of the data. Values higher than the averages are shown in **red** and lower in **blue**, with each row representing discriminant features and each column representing the respective cultivars.

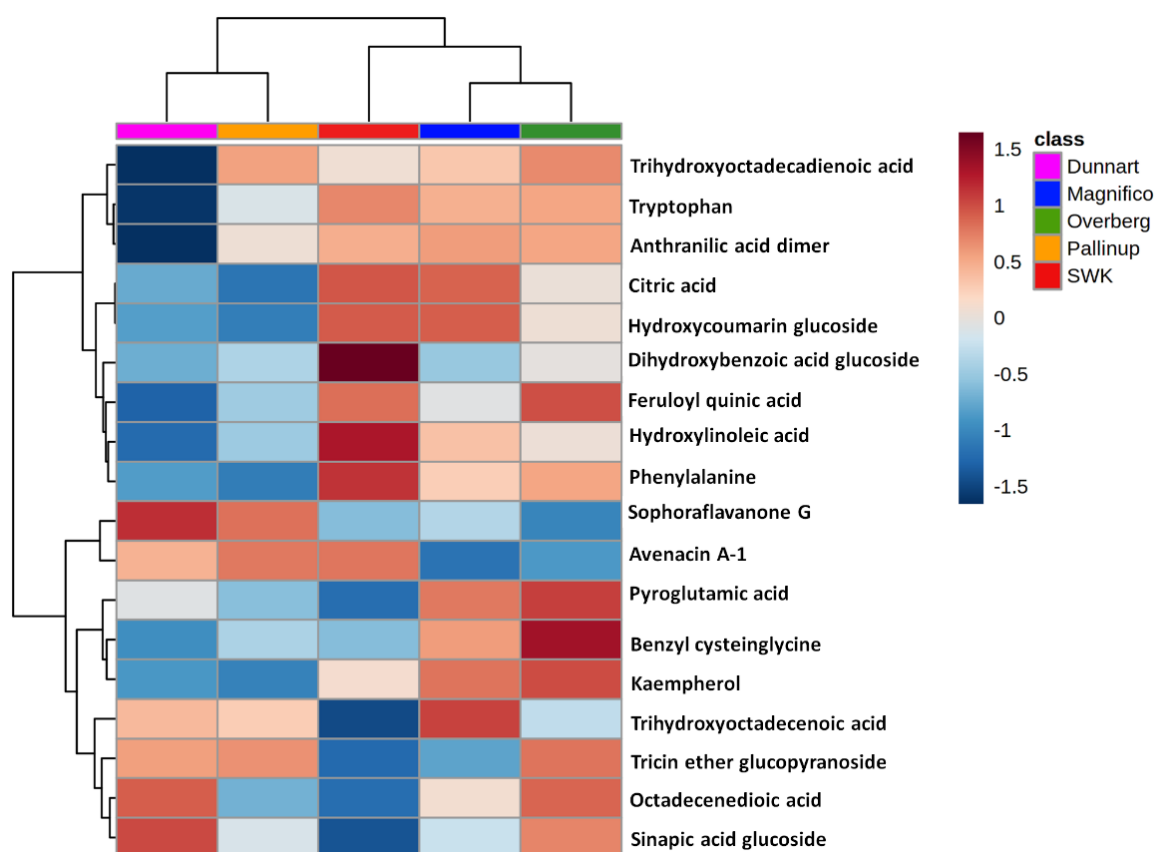


Figure 3.7: Individual peak intensities of putatively identified metabolites from oat roots using heatmap analysis (Pearson distance and Ward's linkage rule) of the five cultivars **Overberg, **Pallinup**, **Dunnart**, **Magnifico** & **SWK001**.** The mean peak intensities of each annotated metabolite are shown after Pareto-scaling of the data. Values higher than the averages are shown in **red** and lower in **blue**, with each row representing discriminant features and each column representing the respective cultivars.

Pathway analyses were performed using the chemometrically extracted metabolites and revealed significant and impactful metabolic pathways. The relative intensities of the different metabolites are illustrated *via* pie charts among the different pathways. Additionally, colour-coded PCA scores plots were used to visually display the presence and abundance of selected discriminant metabolites among the different cultivars (expressed as integrated peak areas in the X data matrix) using vector continuous properties available on SIMCA software (**Supplementary Figure S3.2**). The most significant pathways included: aromatic amino acid (Phe, Tyr, and Trp) biosynthesis, the phenylpropanoid - and flavonoid pathways as well as the stilbenoid biosynthesis pathway. The linoleic acid pathway was illustrated to be most impactful followed by phenylalanine (**Figure 3.8**). Phenylpropanoid metabolic pathways involve some of the most widely occurring plant secondary metabolites which exhibit a range of biological functions involved in the development, defence against biotic and abiotic stresses, and modulation of biochemical processes. Additionally, phenylpropanoids are also important for

the biosynthesis of key compounds like flavonoids, coumarins and lignans (Fraser & Chapple, 2011). Linoleic acids (C18:2) are unsaturated fatty acids that are abundant in plant membranes, thus making them important for plant structure and maintaining water permeability and, additionally, are involved in the synthesis of jasmonates, which act as signalling molecules in response to tissue damage caused by pathogens, insects, herbivores or mechanical stress (Wasternack & Song, 2017).

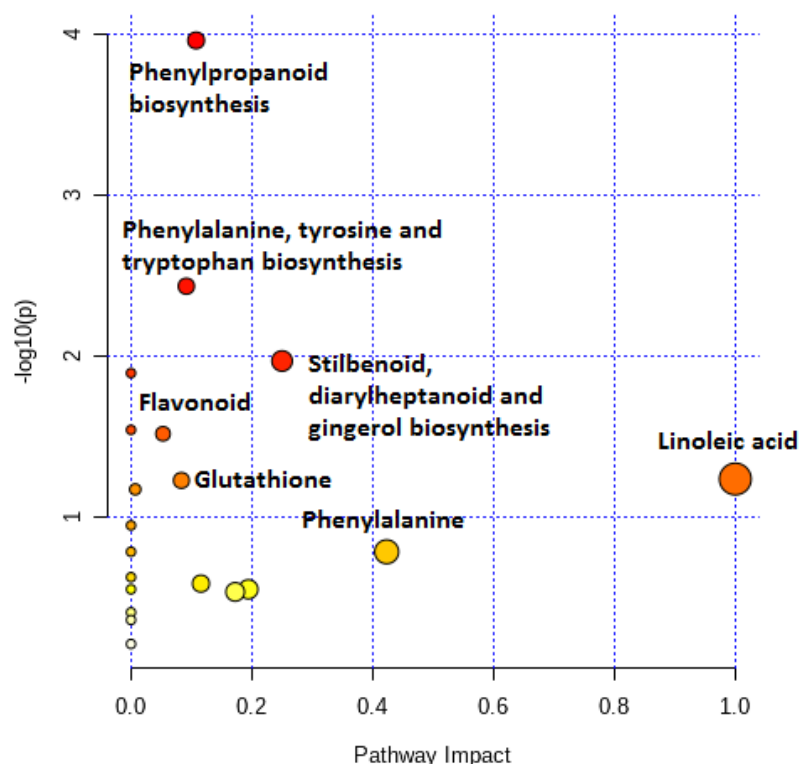


Figure 3.8: Pathway analysis summary of all MetaboAnalyst-computed metabolic pathways displayed according to their significance or pathway impact. The figure illustrates all the matched pathways arranged by p -values (y-axis; pathway enrichment analysis) and the pathway impact values (x-axis; pathway topology analysis). Each node is coloured according to their corresponding p -values, with the node sizes determined according to their impact values. The graph thus illustrates the pathways with high impact: linoleic acid (C18:2, n-6) pathway, phenylalanine, and stilbenoid biosynthesis, and the pathways with high statistical significance: phenylpropanoid, phenylalanine, tyrosine, and tryptophan biosynthesis.

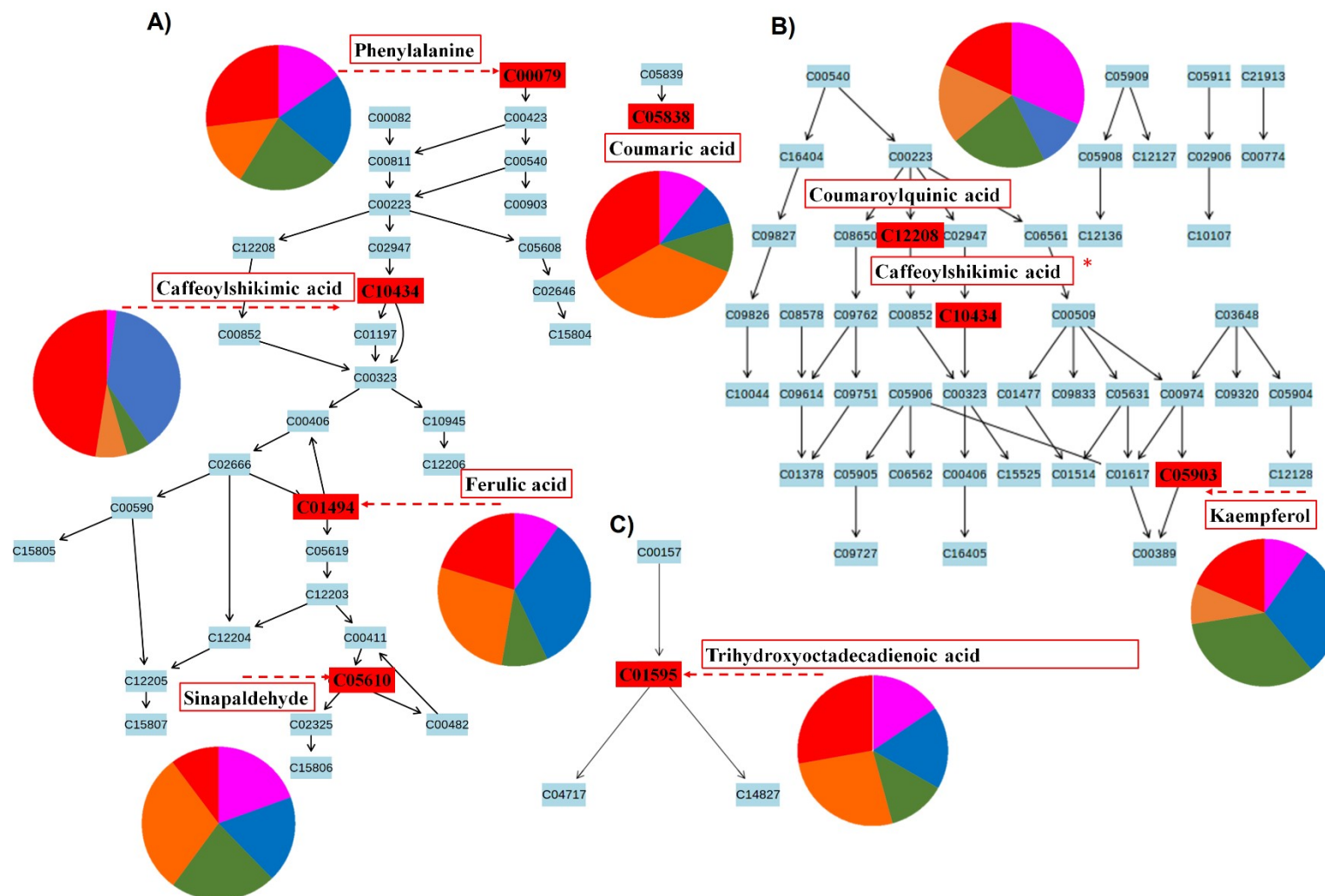


Figure 3.9: Pathways flagged from metabolomics analysis using MetaboAnalyst software. Signatory metabolites involved in each pathway are illustrated in the form of a pie chart according to their relative intensities and presence across the different cultivars. **(A)** Phenylpropanoid pathway, **(B)** Flavonoid pathway overlapping with the phenylpropanoid pathway (*) and **(C)** The linoleic acid pathway that showed the highest impact after pathway enrichment analysis. Some limitations in MetaboAnalyst prevented the mapping of all annotated metabolites (Table 3.1). The different colours indicate the cultivars as before: **Overberg**, **Pallinup**, **Dunnart**, **Magnifico** & **SWK001**.

VIP scores plots (**Supplementary Figure S3 & S4**) were derived from PLS-DA analysis performed in MetaboAnalyst and indicate the key discriminatory metabolites by summarising the weighted sum of squares of the PLS loadings, whilst factoring in the explained Y-variance of each component. Here, metabolites with a VIP score of >0.5 were considered significant in the discrimination between the different cultivars. Some examples can be seen, as in the case with coumaric acid in leaf tissue (**Figure S3**), which presented as a discriminatory feature in the **Pallinup** and **SWK001** cultivars with **Pallinup** showing a greater abundance. Similarly, in the root tissue (**Figure S4**), tricin ether glucopyranoside presented as a discriminatory feature for **Overberg** and **Pallinup** with **Overberg** showing a greater abundance.

The averaged peak intensities of each metabolite were also combined to produce radar charts (**Figure 3.10**), comparatively displaying the metabolomes of the leaf and root tissue. A radar chart is a graphical method used to display multivariate data in a two-dimensional plane and illustrates several quantitative variables on axes originating from the same point. These charts are informative as it sorts the variables into different positions that show distinct correlations between the different groups (Porter & Niksiar, 2018). In the respective radar plots, a range of metabolites is presented and plotted based on their averaged peak intensities. In **Figure 3.10A** clear differences and correlations can be seen as illustrated. Isovitexin 2"-O-glucoside showed to be least abundant in **SWK001** and most abundant in **Dunnart**. Isorhamnetin glucoside, on the other hand, was demonstrated as least abundant in **Dunnart** and most abundant in **SWK001**. These charts are therefore informative in differentiating among the various cultivars based on the respective discriminatory metabolites.

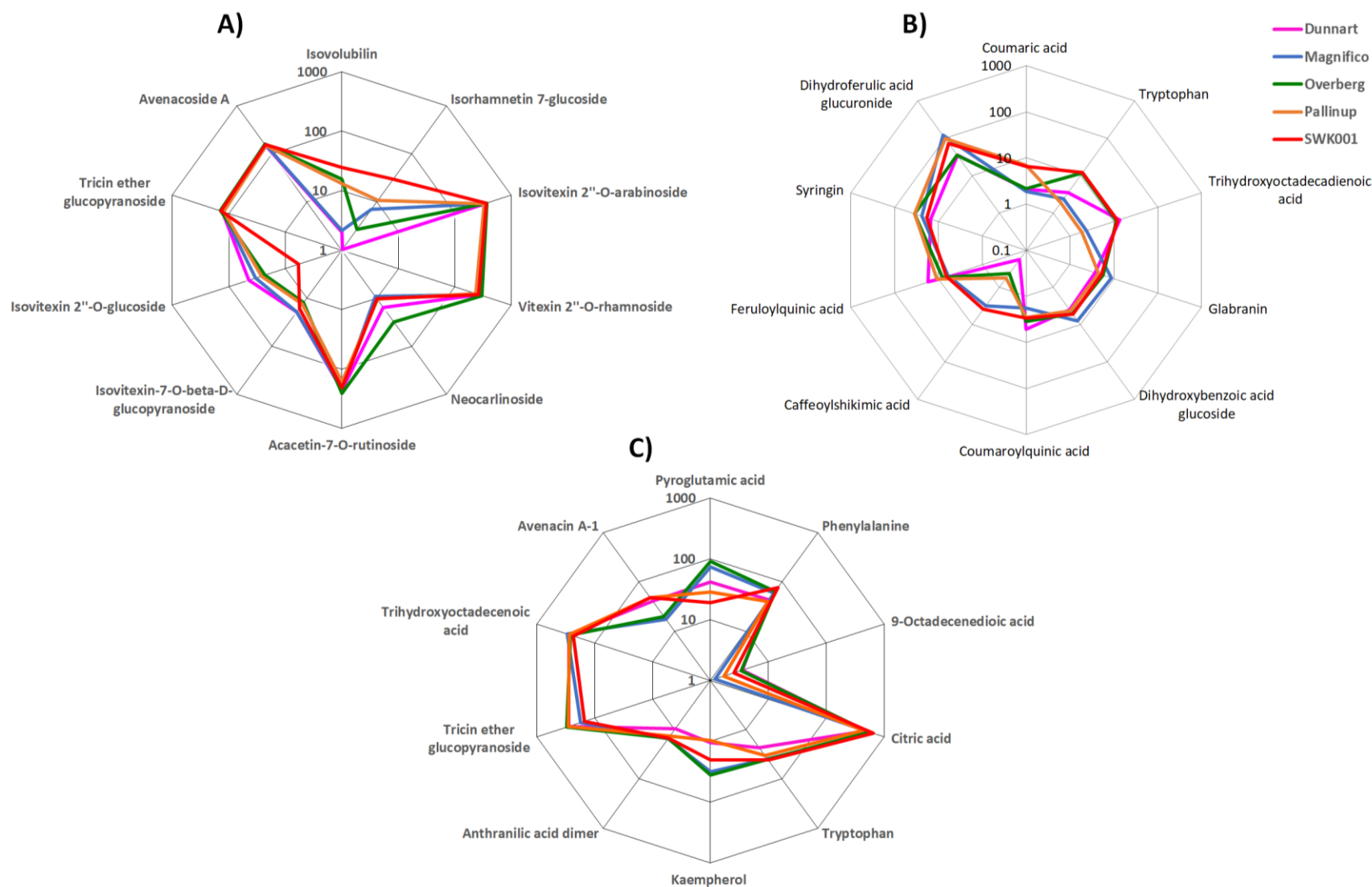


Figure 3.10: Radar charts illustrating relative intensities of the respective metabolites across the different cultivars (Overberg, Pallinup, Dunnart, Magnifico & SWK001) in leaf (A, B) and root (C) tissue. The relative peak intensities were averaged and illustrated as log-transformed values.

To summarise, the results show clear cultivar-related differences with regard to the respective underlying metabolic profiles. Metabolomics as a tool for cultivar discrimination would thus provide a quicker view of the metabolome that could be applied in plant breeding studies to not only differentiate- but also elucidate possible resistance or susceptibility traits among the cultivars. The results show metabolic differences for carboxylic acids, amino acids, fatty acids, phenolic acids (hydroxycinnamic acids and hydroxybenzoic acids and associated derivatives), flavonoids and saponins. **Figure 3.11** summarises the distribution of the discriminatory metabolic markers from the respective metabolite classes (represented are phenolic acids, saponins, flavonoids and fatty acids) and their biological functions. Based on the graphical summary, clear differences and overlap can be seen among the different cultivars. For instance, **Overberg** can be seen as containing a greater number of phenolics and flavonoids as discriminatory markers, therefore this cultivar could exhibit a multitude of beneficial traits related to the presence of metabolites from these classes such as anti-oxidant and anti-pathogenic activity. The **Dunnart** and **SWK001** cultivars can be seen as containing both avenacoside A and avenacin A-1 as discriminatory, that could suggest greater leaf and root defence based on the biological activity of these compounds. Ultimately, these metabolic differences contribute to biological differences and could affect how the respective cultivars respond to abiotic and biotic factors.

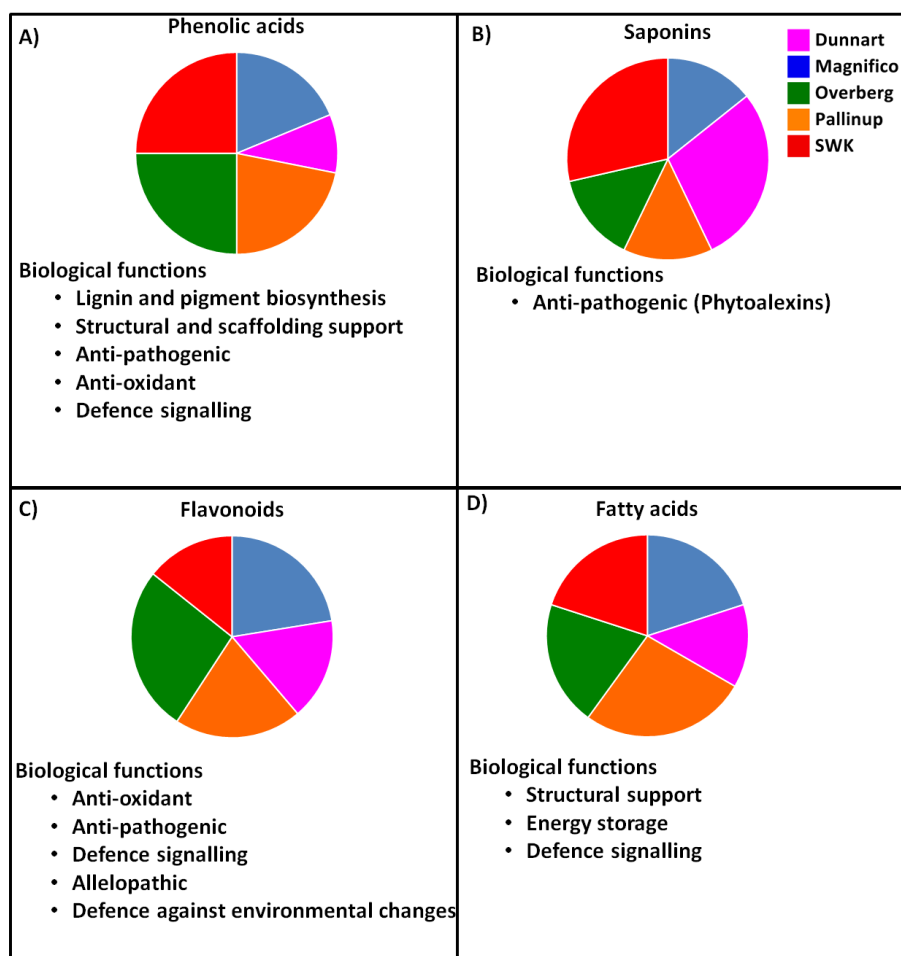


Figure 3.11: The distribution and biological activities of the identified metabolite classes across the respective cultivars (Overberg, Pallinup, Dunnart, Magnifico & SWK001). This figure summarises cultivar-related differences caused by changes in the underlying metabolic profiles of the respective cultivars (leaves and roots). Each pie chart illustrates where a greater number of discriminatory metabolites were identified for each class across the cultivars. **(A)** Phenolic acids- a greater number of metabolites from this class was identified as discriminatory for the SWK001 and Overberg cultivars. **(B)** Saponins (avenacoside A and avenacin A-1)- SWK001 and Dunnart had both saponins from leaf and root present as discriminatory markers for these cultivars. **(C)** Flavonoids- Magnifico and Overberg had a greater number of metabolites from the flavonoid class that presented as discriminatory for these cultivars. **(D)** Fatty acids- Pallinup has the greatest number of metabolites as discriminatory from this particular class of metabolites.

3.4 Conclusion

Metabolomics has been widely applied in crop plant sciences and has shown great progress in understanding how the phenotype links to the metabolome and, by extension, elucidate the active role of metabolites under normal and stress conditions. Metabolomics could therefore provide insights into understanding crop physiology and underlying metabolic events. This could greatly improve crop breeding which is currently based on gene and marker-assisted selection. Although the latter has shown success in crop improvement, it is also faced with

many limitations like the fact that the presence of a gene does not ensure the expression of the trait. Metabolomics has the potential to overcome this limitation and provide useful insights about metabolites involved in resistance, growth, and stress responses which, in turn, can be applied for crop improvement. Thus, in this study, LC-MS-based metabolomics was applied to interrogate the metabolomes of five different oat cultivars. This multidisciplinary omics approach allowed the elucidation and characterization of differential metabolic profiles that define natural variation among oat metabolomes under consideration. The identified metabolic classes were carboxylic acids, amino acids, fatty acids, phenolic compounds (hydroxycinnamic acids and hydroxybenzoic acids and associated derivatives) and flavonoids. Also, a steroidal saponin (avenacoside A) was annotated in extracts from leaves and a triterpenoid saponin (avenacin A-1) from roots. The differences in the metabolic profiles indicate that untargeted metabolomics can be used to distinguish between cultivars. The results further indicate that to discriminate between the different cultivars, the presence or absence of specific metabolites cannot be the only concluding factor, and the relative intensities or ratios of the metabolites also need to be considered as distinguishing criteria. The secondary metabolite classes that were mentioned have various biological roles that are important in plant growth and development, preventing pathogen infections and maintaining the plant under various environmental conditions. Ultimately, an untargeted LC-MS-based metabolomics approach can be used to detect the underlying metabolites that contribute to phenotypic and physiological traits. This will greatly contribute to a more holistic comprehension of the oat plant metabolome which can ultimately be applied in crop improvement and breeding strategies.

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3.5 Supplementary materials

All relevant material referred to, but not included in the main text are included in the following supplementary file. All experimental raw data is available on request at the Department of Biochemistry, University of Johannesburg with permission from Prof. IA Dubery.

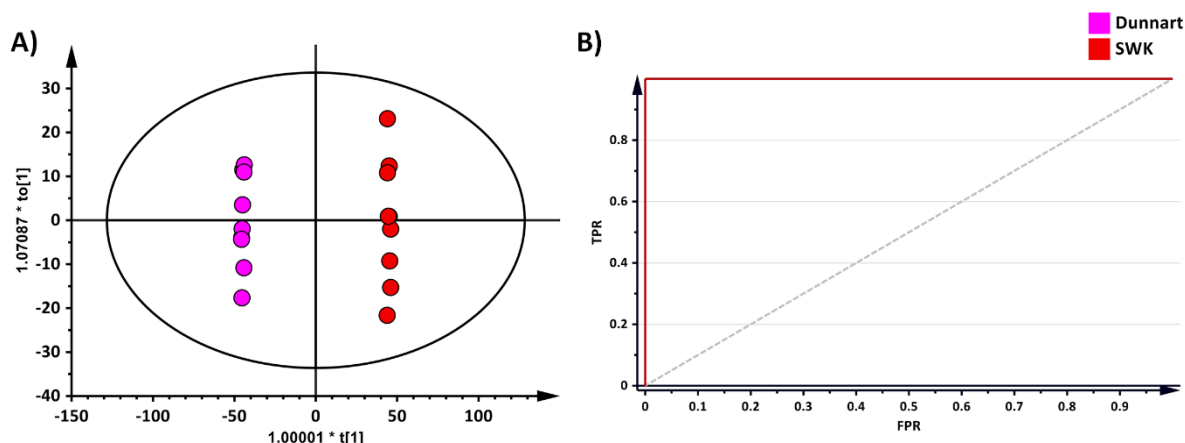


Figure S3.1: An orthogonal projection to latent structures discriminant analysis (OPLS-DA) model of two representative cultivars: Dunnart and SWK001. (A) An OPLS-DA scores plot summarising the relationship among different datasets to visualise group clustering between the Dunnart and SWK001 cultivars based on their leaf-extracted metabolic profiles obtained in ESI(-) MS mode ($R^2=0.998$, $Q^2=0.971$, CV-ANOVA p -value= 5.8941×10^{-14}). (B) Receiver operator characteristic (ROC) plot for the OPLS-DA model (A) illustrating the performance of the binary classifier, showing perfect discrimination (100% sensitivity and 100% specificity) as the curve passes through the top left corner.

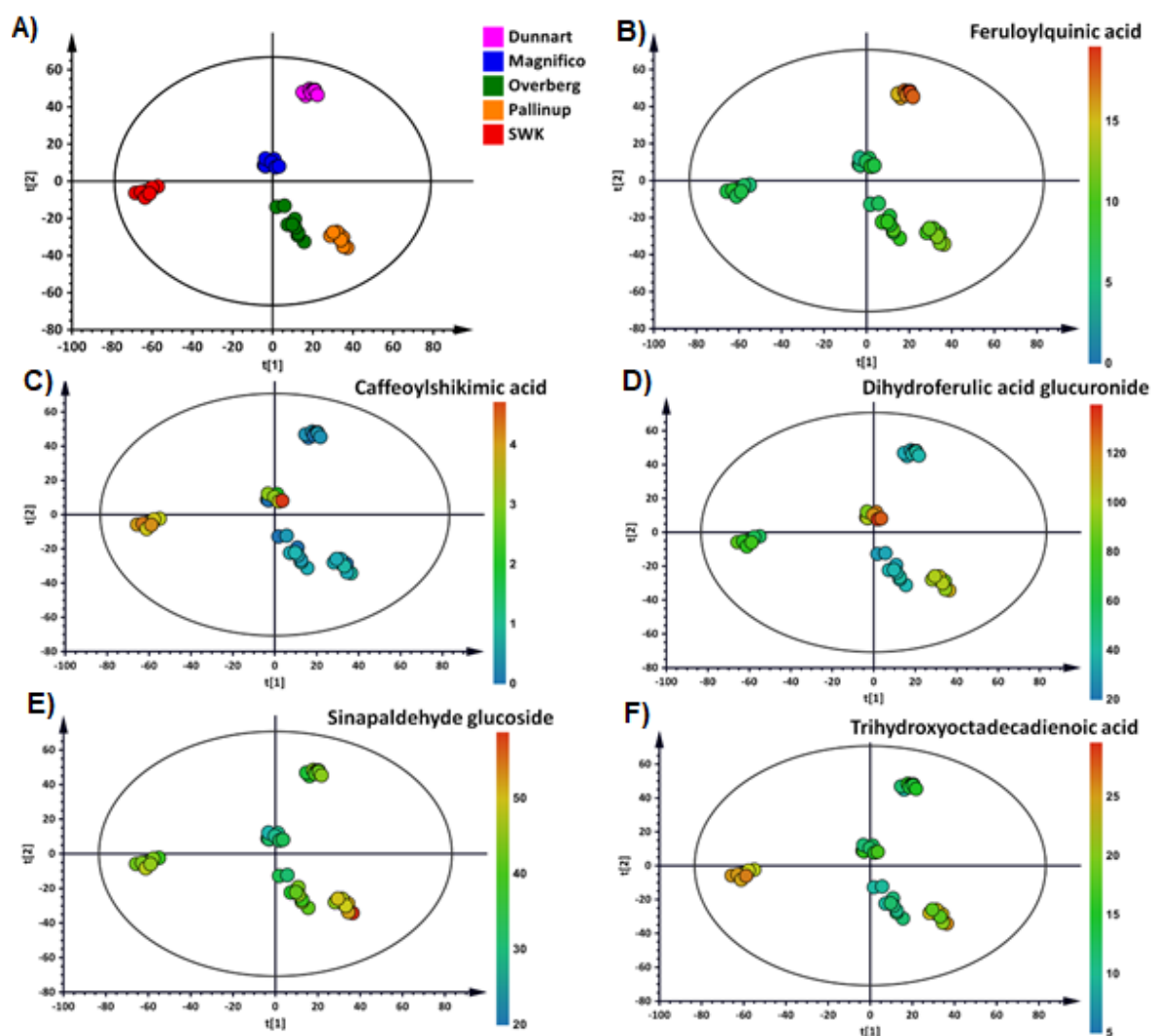


Figure S3.2: Colour-coded PCA score plots showing the presence and increasing abundance of discriminatory ions in the respective cultivars **Overberg**, **Pallinup**, **Dunnart**, **Magnifico** & **SWK001** (red-high abundance, blue-low abundance). (A) PCA scores plot, (B) Feruloylquinic acid, (C) Caffeoylshikimic acid, (D) Dihydroferulic acid glucuronide, (E) Sinapaldehyde glucoside and (F) Trihydroxyoctadecadienoic acid.

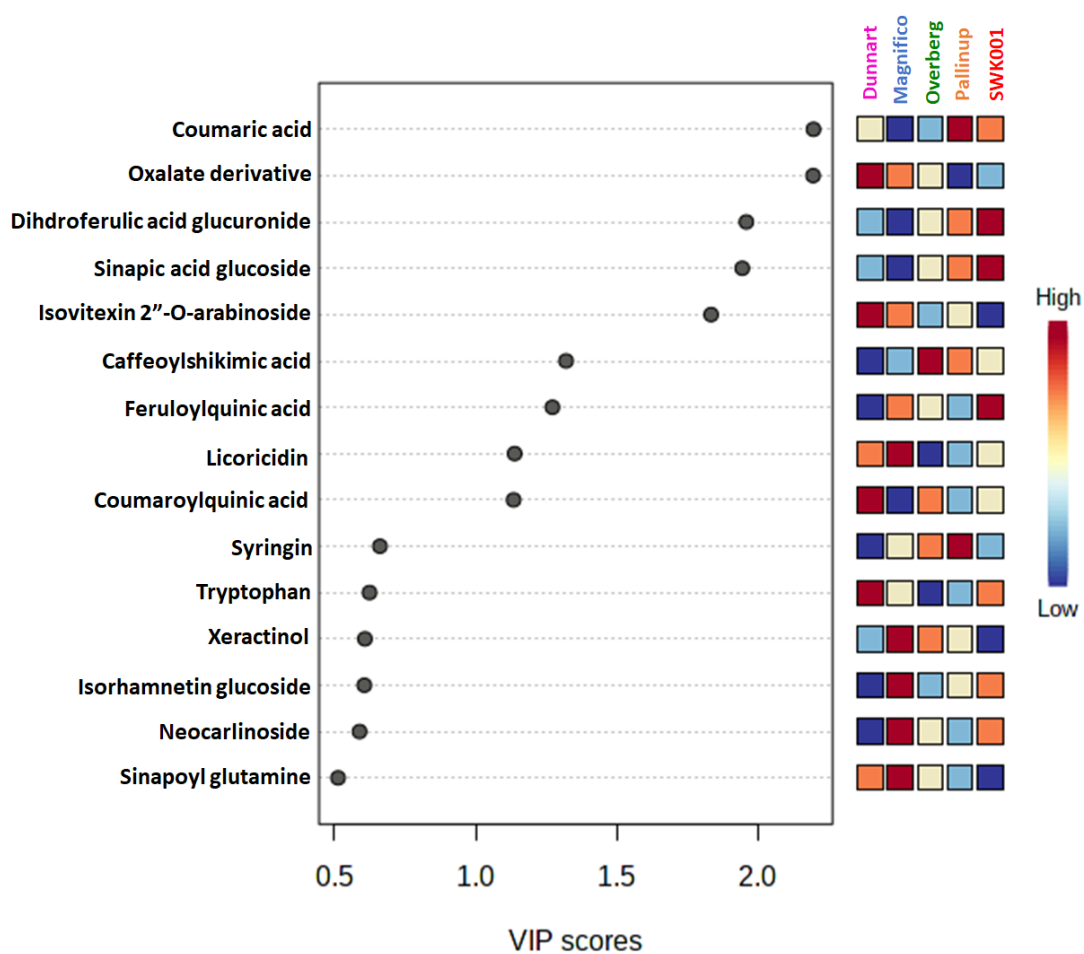


Figure S3.3: Variable importance in projection (VIP) scores generated from partial least squares discriminant analysis (PLS-DA) for the leaves. Indicated are the top 15 discriminating ions in oat leaves from the respective cultivars **Overberg**, **Pallinup**, **Dunnart**, **Magnifico** & **SWK001**. Metabolites with a VIP score ≥ 0.5 were considered to be significant in the discrimination between the cultivars.

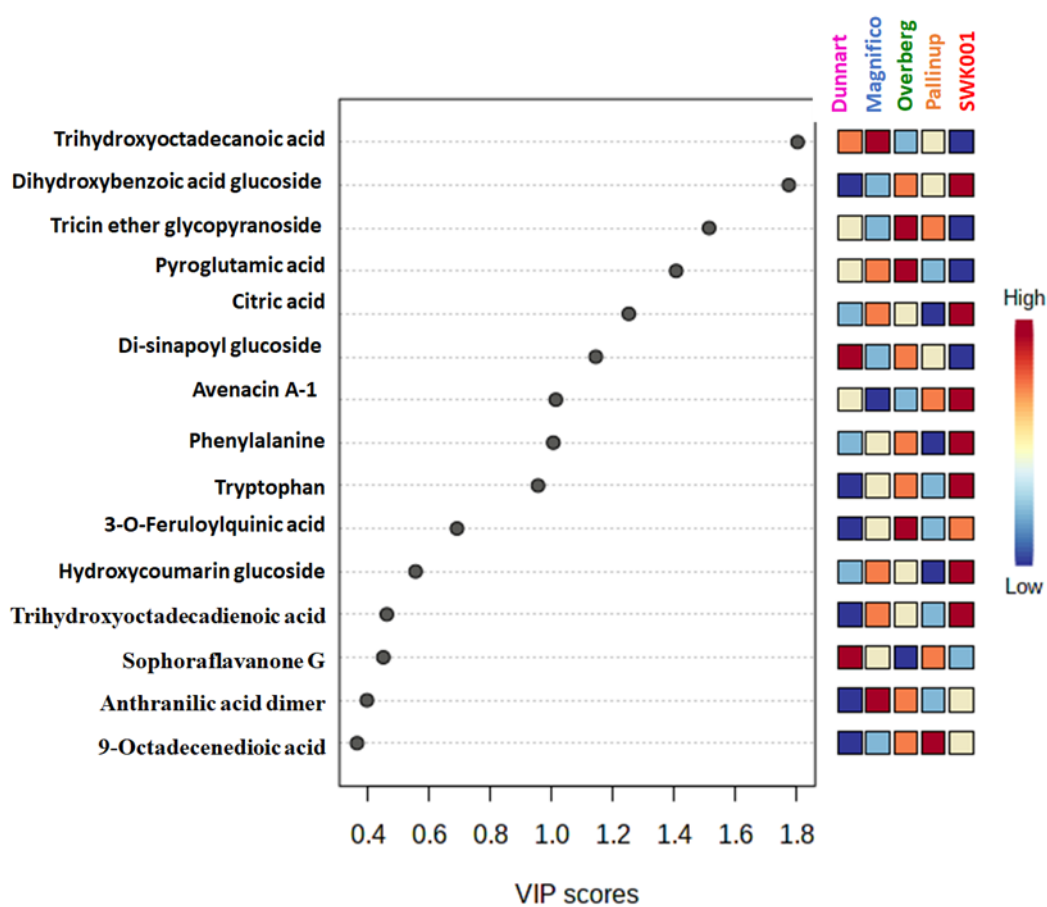


Figure S3.4: Variable importance in projection (VIP) scores generated from partial least squares discriminant analysis (PLS-DA) for the roots. Indicated are the top 15 discriminating ions in oat roots from the respective cultivars Overberg, Pallinup, Dunnart, Magnifico & SWK001. Metabolites with a VIP score ≥ 0.5 were considered to be significant in the discrimination between the cultivars.

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Chapter 4

Metabolomic characterisation of halo blight disease in oat cultivars caused by *Pseudomonas syringae* pv. *coronafaciens*

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Abstract

Background: The metabolome is the underlying biochemical layer of the phenotype and offers a functional readout of the cellular mechanisms involved in a biological system. Since metabolites are considered the end-products of regulatory processes at a cellular level, their levels are regarded as the ultimate response of the biological system to genetic or environmental changes. Hence, the metabolome serves as a metabolic fingerprint of the biochemical events that occur in a biological system under specific conditions. **Aim:** In this study, an untargeted metabolomics approach was applied to elucidate biochemical processes implicated in oat plant responses to *Pseudomonas syringae* pv. *coronafaciens* (*Ps-c*) infection and to identify signatory biomarkers related to defence responses and disease resistance against halo blight. **Methods:** Metabolic changes in two oat cultivars (Dunnart and SWK001) responding to *Ps-c*, were investigated at the 3-leaf growth stage. The cultivars were inoculated with bacterial suspensions and monitored over time: 1, 2, 3, and 4 days post-inoculation. Non-infected plants were used as negative controls. Metabolites were extracted using an 80% methanol extraction method. The extracts were analysed using an ultra-high-performance liquid chromatography (UHPLC) system coupled to a qTOF high definition mass spectrometer analytical platform. **Results:** The acquired multi-dimensional data were processed for multivariate statistical analysis and chemometric modelling. The demonstrated chemometric models indicated time- and cultivar-related metabolic changes that suggest host responses to the bacterial inoculation. Further multivariate analyses of the data were performed to profile differential signatory biomarkers, which included amino acids, phenolics, phenolic amides, fatty acids, flavonoids, alkaloids, terpenoids, lipids, saponins and plant hormones. **Conclusion:** Based on the results, metabolic alterations involved in oat defence responses to *Ps-c* were elucidated and key signatory metabolic markers identified. The study thus contributes toward a more holistic view of the plant metabolism under biotic stress.

4.1 Introduction

Due to their sessile nature, plants have developed a range of defence mechanisms against biotic and abiotic stresses. One such mechanism includes the initiation of adaptive responses such as stress recognition, signal transduction, activation of several stress-related genes accompanied by the production and activation of secondary metabolites or phytochemicals (Onaga & Wydra, 2016). A range of metabolites are readily available and act as constitutive defence compounds prior to the pathogenic attack (phytoanticipins), *i.e.* these preformed compounds occur in healthy plants. Other defence compounds are inducible and released upon pathogen infection, and are known as phytoalexins (González-Lamothe *et al.*, 2009; Gull *et al.*, 2019; Isah, 2019). Changes in plant metabolism are key in understanding and analysing the result of attempted infections. Metabolomic analyses of healthy and newly infected plants, could thus provide invaluable information on changes in signalling or output pathways (Hong *et al.*, 2016; Castro-Moretti *et al.*, 2020). Plant metabolism involves the regulation of different metabolic processes that enable plants to survive environmental threats. The analysis of known primary metabolites (like carbohydrates, amino and organic acids) have been greatly studied to elucidate to what extent plant metabolism is altered to adapt to an ever-changing environment (Fraire-Velázquez & Balderas-Hernández, 2013; Jorge *et al.*, 2016). Conversely, secondary metabolites such as phytohormones (*e.g.* auxins, cytokinins, gibberellins, jasmonates (JA), abscisic (ABA) and salicylic acids (SA), which are key metabolites for plant signalling in response to stimuli from the abiotic/biotic environment, and chemically diverse phytoalexin compounds, synthesised after stimulation by various pathogenic organisms, can be analysed as key components in understanding plant response and defence mechanisms (**Figure 4.1**) (Mazid *et al.*, 2011; Arruda *et al.*, 2016; Wani *et al.*, 2016).

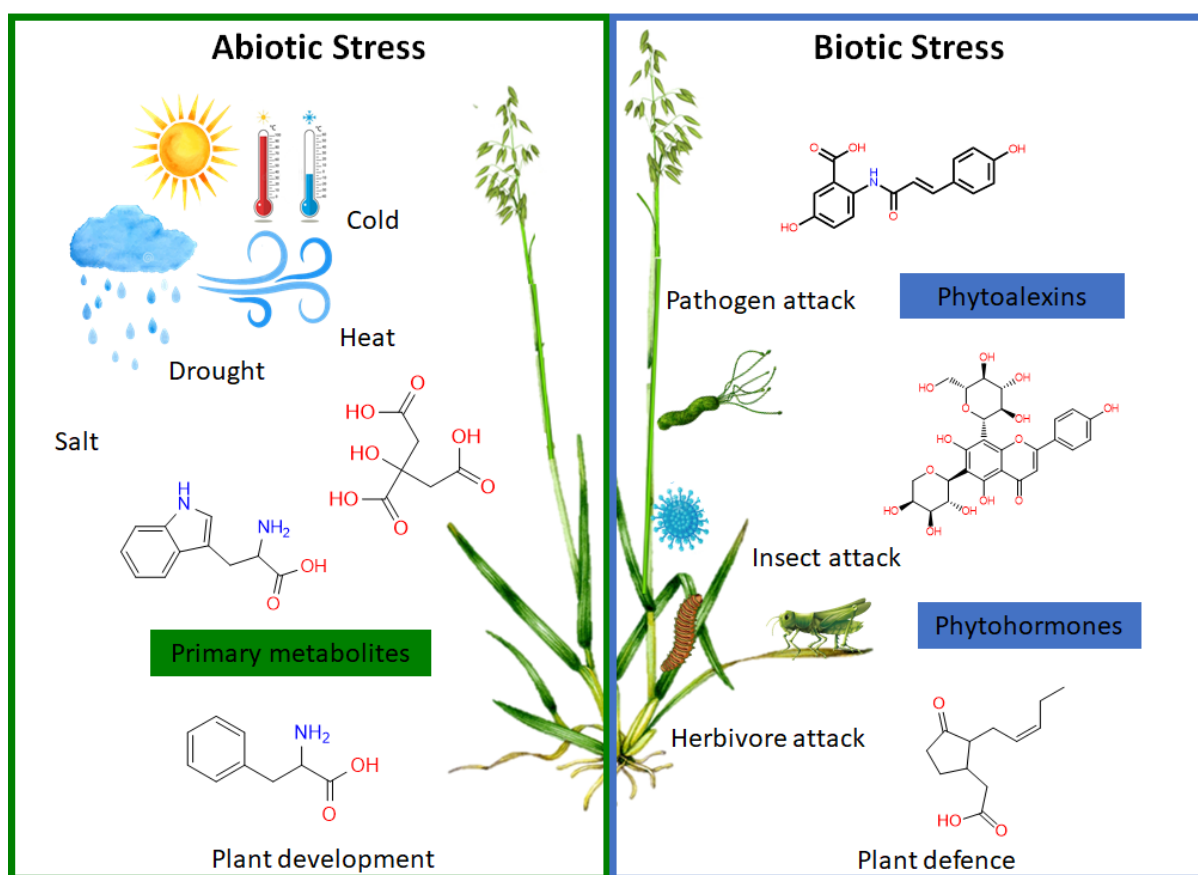


Figure 4.1: Key metabolic components that underlie plant responses to abiotic and biotic stressors. Plant metabolomics is a powerful tool for studying the metabolic mechanisms that trigger and embody plant stress responses to environmental (left) and/or pathogenic (right) threats (adapted from Jorge *et al.*, 2016).

Like all plants, oat crops are often exposed to a range of pathogens that can lead to severe disease and great crop losses. *Pseudomonas syringae* pv. *coronafaciens* (*Ps-c*) is the common cause of halo blight disease in oat crops (Dutta *et al.*, 2018). This pathogen has been reported to impair oat health resulting in yield- and economic losses (Elliott 1920; Wilkie 1972; Harder and Harris 1973; Kim, 2020). It has been known to cause a hypersensitive response (HR) on oat leaves that leads to necrotic spots characterised by small, oval-shaped, water-soaked spots found on leaves in early onset of the disease, and soon change to reddish-brown, oval-shaped lesions with a light centre and a characteristic yellow halo surrounding the lesions. In severe cases of infection, young leaves become curved and chlorotic without the presence of necrotic spots or broad yellow halos (Persson & Sletten, 2001). Environmental conditions can affect disease severity, development and spread of halo blight disease as *Ps-c* favours moist conditions and optimally spreads through rain, wind and insects (Martinelli, 2000; Lamichhane *et al.*, 2014).

The general fundamental concepts of plant innate immunity have been well established (Jones and Dangl, 2006), and involves recognition of pathogen-derived molecular pattern molecules by cell surface located receptors as well as intracellular recognition of pathogen effector proteins by resistance (R) proteins from the plant host (**Chapter 2**). This implies a strong genetic basis for disease resistance or susceptibility, that is ultimately reflected in the metabolomic phenotype (**Chapter 3**). Here an untargeted LC-MS-based metabolomics approach was used to detect underlying metabolic alterations and identify potential metabolic biomarkers that contribute to oat defence in response to inoculation with *Ps-c*. While some oat cultivars exhibit a higher level of disease resistance, the molecular mechanisms underlying these interactions are still poorly understood (Gorash *et al.*, 2017). By analysing the cellular and molecular responses between the plant and pathogen, sustainable means of combating disease could be developed and be particularly useful in breeding programs by identifying metabolic markers associated with resistance or susceptibility (Kumar *et al.*, 2017).

4.2 Materials and Methods

4.2.1 Oat plant cultivation

Seeds of the oat cultivars **Dunnart** (Agricol, Pretoria, South Africa) and **SWK001** (ARC Small Grain Institute, Bethlehem, South Africa) were selected for infection through an initial screening trial (**section 4.2.2**). Seedlings were grown in 10 cm pots (15 seeds per pot) containing germination mixture (Culterra, Muldersdrift, South Africa), and watered twice a week. The plants were grown under greenhouse conditions: a light/dark cycle of 12 h/12 h, with a light intensity of about 84 $\mu\text{mol}/\text{m}^2/\text{s}$ and temperature between 25–28 °C. The study was designed to monitor the response of these cultivars to bacterial infection over time, 1-4 days post-inoculation (d.p.i). The seedlings were grown in triplicate as biological replicates (one pot = one biological replicate) for every time point and the corresponding control groups under the same environmental conditions. Once the plants reached 3-week maturity (seedling stage or three-leaf stage), they were infected (**section 4.2.3**). The entire experiment was repeated twice.

4.2.2 Preparation of *Pseudomonas syringae* pv. *coronafaciens*

A culture/strain of *Pseudomonas syringae*, pathogenic on oat, was isolated by Dr. W. Kriel (Starke Ayres Seeds, Bredell, South Africa) and its identity as *P. syringae* pv. *coronafaciens* (based on 16S rRNA sequencing) was confirmed by Prof. T. Coutinho (Centre for Microbial Ecology / Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa). This *Ps-c* isolate was then grown and maintained on nutrient agar. A colony was picked under sterile conditions in a laminar flow cabinet and grown overnight at 28 °C in nutrient broth on an orbital shaking incubator. The OD₆₀₀ of the overnight culture was measured and diluted with 0.1 % Tween 20 and phosphate buffered saline (PBS) to an OD₆₀₀~ 0.3. The same dilution was made for non-inoculated nutrient broth to serve as a medium control.

The virulence of the *Ps-c* strain on the oat cultivars was investigated using drop-inoculation (Hiruma & Saijo, 2016) on leaf segments (OD₆₀₀ = 0.1, 0.2, 0.3). The inoculated leaf segments were kept in a container with high humidity for 5 d. The selection of **Dunnart** and **SWK** for further metabolomic investigation as cultivars exhibiting a resistance response vs. a susceptible response, was based on initial visual observation studies that showed **Dunnart** being able to tolerate the infection over the course of 5 d, and **SWK** showing symptoms and susceptible characteristics only 2 d after drop-inoculation, respectively.

4.2.3 Inoculation of oat seedlings

At the three-leaf growth stage, the oat plant leaves were treated by spraying with the *Ps-c* bacterial suspension (0.1 % Tween 20 and PBS), diluted to OD₆₀₀~ 0.3. The non-treated control plants were sprayed with a solution free of the bacteria and the negative control groups were untreated (i.e. not sprayed with either solution) and grown under normal growth conditions. The 50 mL of either the inoculum (containing the bacteria) or the control (0.1% Tween 20 and PBS) solution was evenly sprayed onto the leaves of the treated and non-treated control groups respectively. The plants were then incubated in darkness in an incubator for 1 h to provide 100% relative humidity. Following the 1 h incubation, the plants were removed and another 50 mL of either inoculum or control solution was applied to the treated and non-treated control groups, respectively, and further incubated for 6 h. After incubation the plants were then exposed again to the same initial conditions: with cycles of 12 h light/dark, a light intensity of

about 84 $\mu\text{mol}/\text{m}^2/\text{s}$ and temperature between 25–28 °C. Post-treatment harvesting of plants was done for treated, non-treated and negative control groups at 1, 2, 3 and 4 d.p.i. by cutting the leaves and immediately snap freezing with liquid nitrogen to quench metabolic activity associated with possible wounding and handling of the tissue. Leaves were stored at –80 °C until metabolite extraction.

4.2.4 Metabolite extraction and sample preparation

Liquid nitrogen was added to the leaf material, which was then crushed into a powder form using a mortar and pestle. One gram per sample was weighed into a clean Falcon tube and 10 mL of 80% cold aqueous methanol (4 °C) was added (m/v ratio of 1:10). The methanol used was of analytical grade (Rochelle Chemicals, Johannesburg, South Africa). The mixture was then homogenised using a probe sonicator (Bandelin Sonopuls, Berlin, Germany) set to 55% power for 10 s per sample. Equipment was cleaned between samples to prevent cross-contamination. The homogenates were centrifuged at 5100 $\times g$ for 20 min at 4 °C in a benchtop centrifuge after which the supernatants were kept and concentrated by evaporating the methanol under vacuum to approximately 1 mL using a rotary evaporator set to 55 °C. The concentrated samples were transferred to 2 mL microcentrifuge tubes and dried in a centrifugal evaporator under vacuum. The dried extracts were then reconstituted by dissolving in 500 μL of 50% aqueous methanol (LC-grade, Romil Pure Chemistry, Cambridge, UK). The samples were subsequently filtered through nylon syringe filters (0.22 μm) into chromatography vials fitted with 500 μL inserts, capped, and kept at 4 °C until analysis.

4.2.5 Sample analyses using ultra-high-performance liquid chromatography (UHPLC)

An Acquity UHPLC system (Waters Corporation, Manchester, UK) was used to analyse 2 μL of each sample, separated into its respective components using a binary solvent on an HSS T3 reverse-phase column (Waters Corporation, Billerica, MA, USA; 2.1 $\times$ 150 mm $\times$ 1.7 μm), thermostatted at 60 °C. The solvents used were MilliQ water and acetonitrile (Romil Chemistry, Cambridge, UK), both containing 0.1% formic acid (Sigma, Munich, Germany) and 2.5% isopropanol (IPA, Romil, Cambridge, UK). The run was set to 30 min per 2 μL injection with an elution gradient carried out *via* a binary solvent system consisting of 0.1% aqueous formic acid (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 95% A and 5% B and held for 1 min. A gradient was applied to change the chromatographic conditions to 10% A and 90% B at 25 min; and changed to 5% A and 95% B at 25.10 min. These conditions were held for 2 min and then changed to the initial conditions at 28 min. The analytical column was allowed to equilibrate for 2 min before the next injection. Pooled quality control (QC) samples were also prepared to monitor the condition of the LC-MS system and assess the reliability and reproducibility of each analysis (Broadhurst *et al.*, 2018). Additionally, blank samples (50% MeOH) were also randomly included in the run to monitor the background noise. Each sample was analysed in triplicate (analytical/technical replicates), and together with the three biological replicates, this generated n=9 for each experiment, to account for analytical variability and have the minimum required number of replicates for metabolomic studies that involve multivariate analyses.

4.2.6 Quadrupole time-of-flight mass spectrometry (q-TOF-MS)

A high definition SYNAPT G1 Q-TOF mass spectrometry system (Waters Corporation, Manchester, UK) was coupled to the chromatography system to detect metabolites and acquire data in both positive and negative electrospray ionisation (ESI) operation modes as previously mentioned in **section 3.2.4**. The controlling software was MassLynx XSTTM (Waters, Manchester, UK). A reference calibrant, leucine enkephalin (554.2615 Da) was used as the 'lockmass' calibrant and allowed for typical mass accuracies between 1 to 3 mDa. The respective capillary and sampling cone voltages were set as 2.5 kV and 30 V. The desolvation temperature used was 450 °C, with the source temperature set to 120 °C, cone gas flow was set to 50 L/h, and the desolvation gas flow set to 550 L/h. An *m/z* range of 50–1200 was set with a scan time of 0.1 s. The desolvation-, collision- and cone gas used at a flow rate of 700 L/h was high-purity nitrogen. Data was acquired using five different collision energies (MS^E), ramping from 0-50 eV to cause fragmentation of the initial ions to ensure that as much information regarding the structures of the respective compounds could be obtained for downstream structural elucidation and metabolite annotation (Tugizimana *et al.*, 2014; Zeiss *et al.*, 2018).

4.2.7 Data analysis

The data sets obtained were explored and processed using the applications manager, MarkerLynx XSTM (Waters Corporation, Manchester, UK). The software makes use of a patented ApexTrack algorithm. The following parameters were used for processing: retention time (Rt) range 2–23 min and m/z range 50–1200 Da. The Rt window was set to 0.20 min and the mass window to 0.05 Da. The mass tolerance was 0.05 Da and the intensity threshold was set to 100 counts. The corrected data matrices were exported to ‘soft independent modelling of class analogy’ (SIMCA-version 15) software (Umetrics, Umea, Sweden) for multivariate data analysis (MVDA). Unsupervised models, namely principal component analysis (PCA) and hierarchical clustering analysis (HCA) were used to reduce the dimensionality of the data sets and to explore the underlying structures and characteristics of the data. Supervised orthogonal projection to latent structures discriminant analysis (OPLS-DA) was used to compare the two cultivars and identify discriminatory ions. The OPLS-DA models were validated using rigorous validation methods that included cross-validated analysis of variance (CV-ANOVA) and receiver operator characteristic (ROC) analysis (Trygg *et al.*, 2007; Tugizimana *et al.*, 2013; Worley & Powers, 2013).

4.2.8 Metabolite annotation and semi-quantitative comparison

Metabolites were identified based on their respective masses, fragmentation patterns, and possible elemental compositions generated from the MarkerLynxTM software. Each putatively suggested empirical formula was exported and searched for in various databases such as MetaCyc (<https://metacyc.org/>), Plant metabolic network (PMN) (<https://plantcyc.org/>), ChemSpider, Mass bank of North America (<https://mona.fiehnlab.ucdavis.edu/>), Dictionary of Natural Products (www.dnp.chemnetbase.com), and the Kyoto Encyclopedia of Genes and Genomes (KEGG, www.genome.jp/kegg/). Metabolites were putatively identified to level 2 of the Metabolomics Standards Initiative (MSI) unless specified otherwise (Sumner *et al.*, 2007).

The generated matrixes of the annotated metabolites were exported from MarkerLynx XSTM as .csv files. MetaboAnalyst 4.0, an online platform for statistical, functional and integrative analysis of metabolomics data (www.metaboanalyst.ca) was then utilised for visualisation of the MS files which contained the m/z , Rt and peak intensities of metabolites separated by chromatography and detected as ions by mass spectrometry. Data processing, integrity, missing

values, filtering and normalisation were performed on MetaboAnalyst followed by Pareto-scaling before statistical analyses to reduce variance within the features. A comparison of the magnitude and presence of the identified metabolites among the various cultivars and treatments were performed *via* heatmap analyses using a Pearson distance measure and Ward clustering algorithm (www.metaboanalyst.ca) (Xia *et al.*, 2009; Chong *et al.*, 2018). Partial least square-discriminant analysis (PLS-DA) was also used to mine the data *via* MetaboAnalyst for the comparison and visualisation of the relative abundances of the identified metabolites across the various cultivar treatments. VIP scores plots, derived from the generated PLS-DAs, indicates the key discriminatory metabolites with a VIP score of >0.5 which are considered significant in discriminating between the *Ps-c* treatment and controls among the two cultivars. Additionally, to further visualise changes among the discriminatory metabolites associated with the defence responses of the two cultivars, radar/radial plots were constructed based on the averages of the relative intensities and illustrated as log-transformed values (**section 4.3.4**).

4.3 Results and Discussion

4.3.1 Disease severity and symptom development of halo blight in oat cultivars

The disease severity and development of symptoms in the **Dunnart** and **SWK001** cultivars (**Figure 4.2 & 4.3**) show cultivar-related differential interactions among the oat cultivars and the *Ps-c* pathogen. Halo blight disease was evaluated by visual observation using a 0 - 8 scale, where 0= no disease symptoms, >1-3= slight disease symptoms, >3-6= moderate disease symptoms and >6-8= severe symptoms of yellowing and wilting (**Figure 4.2**) (Muedi *et al.*, 2015). **Dunnart** showed typical halo blight symptoms such as the small water-soaked spots on the surfaces of the leaves. As the symptoms developed a characteristic yellow halo appeared around the spots (**Figure 4.3A**). This halo is due to the action of a toxin produced by the bacteria and is a diagnostic symptom of the disease (An *et al.*, 2015). In severe infections, symptoms commonly include the leaves and upper parts of the plant turning yellow (chlorotic) and shrivelled (Harveson *et al.*, 2007) as can be seen with the **SWK001** cultivar (**Figure 4.3B**).

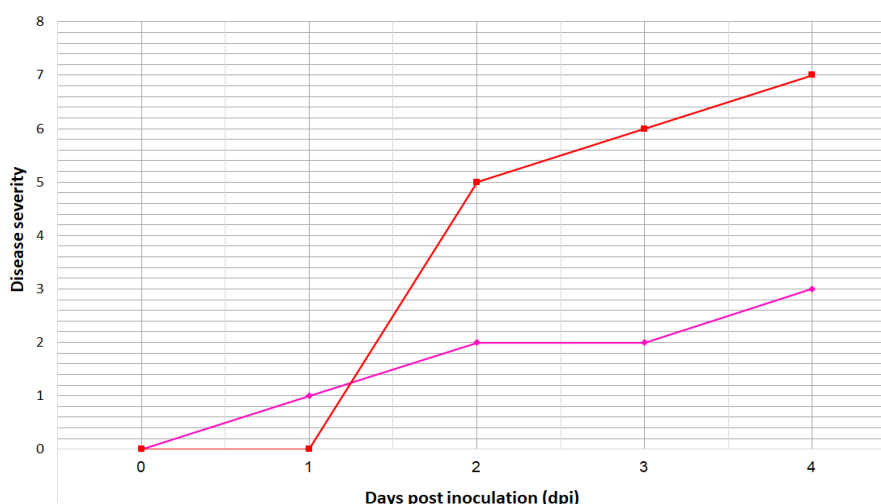


Figure 4.2: Disease severity rating for oat cultivars responding to *Ps-c* infection over a period of 4 days post-inoculation (d.p.i.). Disease severity scores for phenotypical symptom development in **Dunnart** and **SWK001** cultivars ranging from 0 = Symptomless (leaves are free of any visual symptoms), 1 = Very limited amount of lesions start appearing (5 % of leaves start showing visual symptoms), 2 = Limited amounts of lesions are present (6%-11% of leaves start showing visual symptoms), 3 = Moderate amounts of lesions are present (11%-20% of leaves start showing visual symptoms), 4 = Severe amounts of lesions are present (21%-50% of leaves start showing visual symptoms), 5 = Limited wilting occurs (6%-11% of leaves become yellow and wilted), 6 = Moderate wilting occurs (11%-20% of leaves become yellow and wilted), 7 = Severe Wilting (21% to 50% of leaves become yellow and wilted) and 8 = Very severe wilting occurs (50% or more of leaves are yellow and wilted).

The phenotypic symptoms and observations can be understood as **Dunnart** exhibiting a stronger defence response than **SWK001**, with HR-like lesions and yellow halo formation around the infected tissue (**Supplementary Figure S4.1 & Figure 4.3A**). In contrast, the **SWK001** cultivar showed little or no resistance against *Ps-c* as this particular cultivar developed severe symptoms (**Figure 4.3B**). The development of halo blight and its severity in oat crops differ depending on the interaction between the pathogen and plant, and the environmental conditions (Saini *et al.*, 2019). In this study the oat cultivars were infected under a controlled environment, and symptomatic differences thus reflect cultivar-related responses to the *Ps-c* infection. It is therefore important to identify the specific variety or cultivar, as great variation may occur within cultivars from the same species with regard to their susceptibility or resistance to specific diseases (Riley *et al.*, 2002). It is well known that plants depend on an inherent complex and multi-layered innate immune system to survive changing environments and pathogenic threats (Tugizimana *et al.*, 2018). Therefore, characterising the metabolic phenotypes associated with oat defence responses to *Ps-c* infection would provide more insight into the cellular and metabolic pathways involved in the plant-pathogen interaction (Preston, 2017).

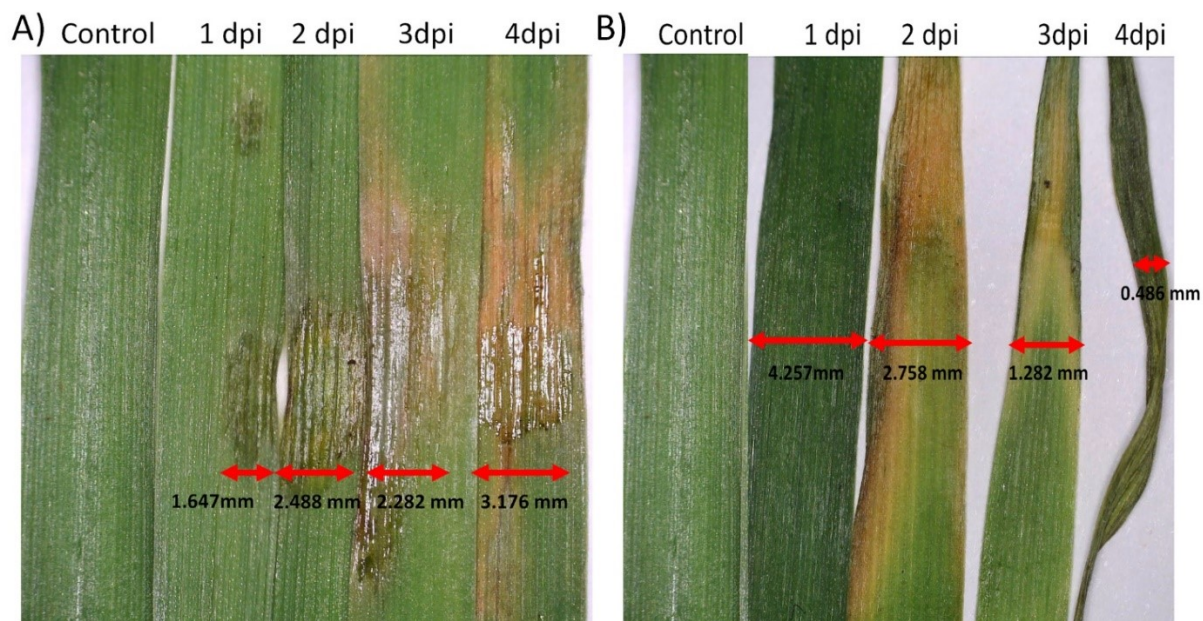


Figure 4.3: Typical symptom development of halo blight on oat leaves in response to *Ps-c* infection. Following inoculation, the development of lesions on the (A) **Dunnart** leaves were observed over time. Lesions started appearing from 1 d.p.i. and spread progressively over time. At 4 d.p.i. the lesions appear to cover a larger section of the leaf surface. In the (B) **SWK001** cultivar severe symptoms can be seen as the leaves start yellowing from the tips at 2 d.p.i. and become more severe over time. At 4 d.p.i. the leaves start dying and appear shrivelled. These symptom development patterns were representative for the **Dunnart** and **SWK001** cultivars respectively.

4.3.2 Liquid chromatography-mass spectrometry-based analyses of oat response to *Ps-c*

Methanolic leaf extracts of the infected, non-infected (vehicle control) and negative control oat plants were analysed on a reversed-phase UHPLC-QTOF-MS system with electrospray ionisation (ESI) in both positive and negative ionisation modes. Initial optimisation studies indicated that the majority of extractable metabolites ionised better in the ESI (-) mode; accordingly, only these data sets are further presented and illustrated. An untargeted approach was used to elucidate and identify as many statistically significant metabolites as possible. Based on the innate biochemical diversity of plants and the multi-dimensionality of the extracted metabolomics data, chromatographic separation provides crucial information as it separates the various components based on their polarity to provide high resolution separation of sample constituents (Tugizimana *et al.*, 2013; Alonso *et al.*, 2015). The coupling of chromatography to mass spectrometry provided a highly sensitive analytical platform that permitted the simultaneous detection of a range of metabolites to provide a more holistic understanding of the metabolic composition of the biological samples (Jorge *et al.*, 2016). **Figure 4.4** illustrates the base peak intensity (BPI) chromatograms with differential peak populations showing the differences and similarities between the infected and non-treated

control groups for both cultivars. The examples illustrated by the chromatographic separation as changes between the infected and non-treated control groups include the increasing abundance of avenanthramide A and L in the infected **Dunnart** cultivar compared to the control group from 1-4 d.p.i (**Figure 4.4A**), and increasing abundance of avenanthramide L in the infected **SWK001** cultivar from 1-4 d.p.i. compared to the control (**Figure 4.4B**). To gain deeper insights into the underlying biochemical changes related to the oat response upon treatment with *Ps-c*, the complex, multi-dimensional data sets were further analysed using chemometrics and multivariate data analysis.

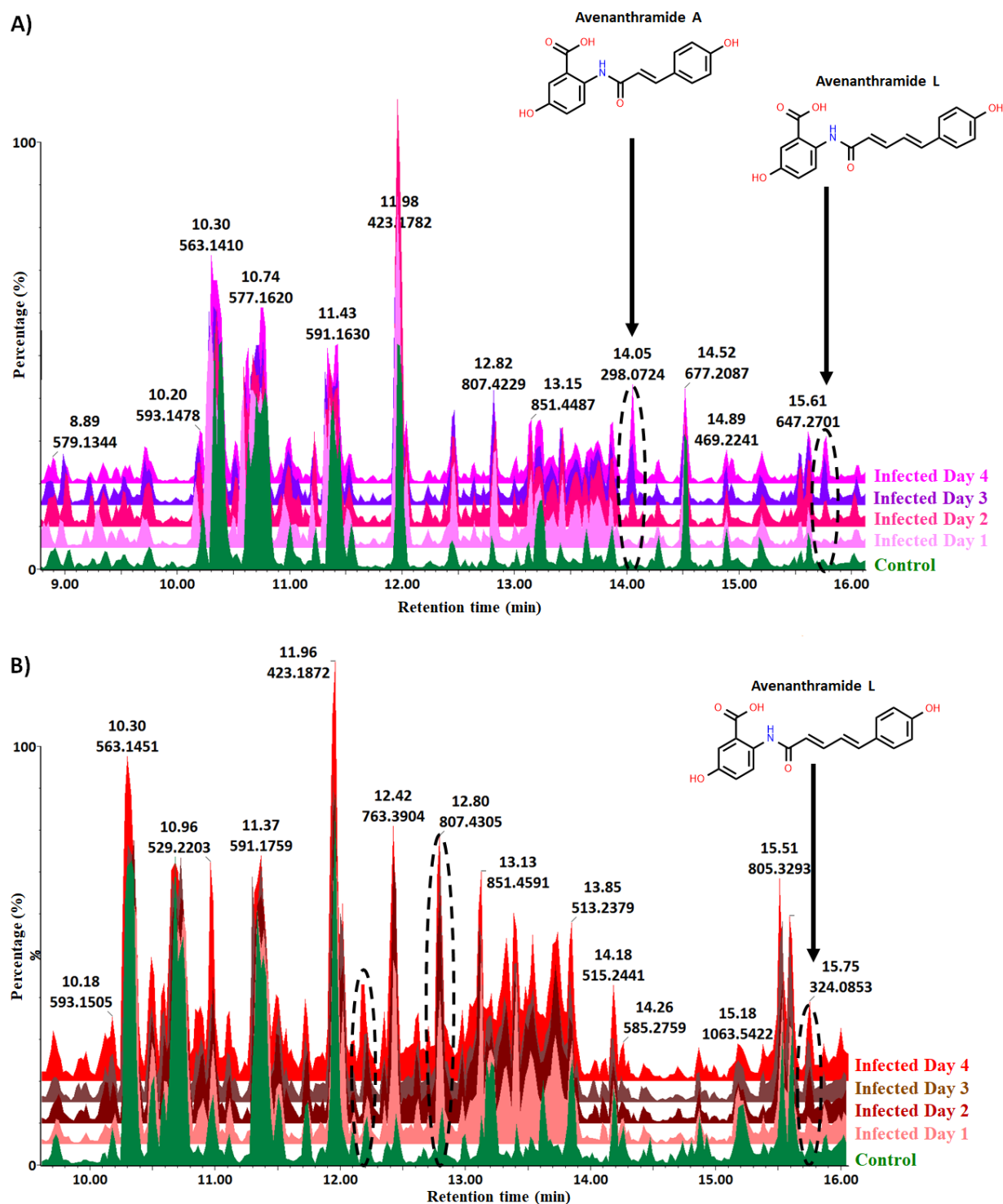


Figure 4.4: Ultra-high-performance liquid chromatography (UHPLC) coupled to mass spectrometric (MS) detection. The figure compares base peak intensity (BPI) MS chromatograms of (A) *Dunnart* infected and (B) *SWK001* infected extracts at the seedling stage compared to the non-treated control (uninfected plants). These represent the chromatographic separation based on the polarity of the different compounds, separated on an HSS T3 reverse-phase column, and ionised with ESI negative mode. The dashed oval structures point out some exclusive variations that illustrate differences in the phytochemical profiles of the infected cultivars over time. As illustrated, avenanthramide phytoalexins were shown to be absent in the control(s) and increase in abundance over time and, although only avenanthramide A and L are shown, four different avenanthramides (A-C and L) were detected.

4.3.3 Chemometrics for the analyses of halo blight induced metabolic changes in oat cultivars

As described in **Chapter 3, section 3.3.2**, PCA is a multivariate technique that is used to explore complex datasets by reducing the multi-dimensionality of the data and thereby revealing structures, trends or groupings that allows for biological interpretability of the data (Jolliffe & Cadima, 2016). Here, PCA modelling revealed the underlying structures and characteristics of the data through the principal components (PCs) that illustrated treatment- and cultivar-related groupings (**Figure 4.5A & C**). The sample groupings point to differential metabolic changes in the **Dunnart** and **SWK001** cultivars in response to *Ps-c* infection. The **Dunnart** cultivar clustered separately from **SWK001** in both the infected and non-treated control groups, again reiterating treatment and cultivar-related differences (**Figure 4.5A**). The negative controls (refer to **section 4.2.3**) clustered with the vehicle controls (**Supplementary Figure S4.2**), meaning that there were no significant differences between the two control groups i.e. the underlying metabolic profiles are similar compared to the infected groups. Therefore only the non-infected vehicle control groups are further presented throughout the study and referred to only as control. **Figure 4.5B** illustrates the PCA model showing differential clustering between the two treated groups (**Dunnart** and **SWK001**) over time (1-4 d.p.i.). HCA graphically presents clusters of the high-dimensional data in the form of a dendrogram based on dissimilarity and similarity between the samples (Zhang *et al.*, 2017). In this study it was used to evaluate whether groupings emerge from the data based on treatment and/ or cultivar-related differences. The bottom-up representation (**Figure 4.5B & D**) uses an algorithm to cluster each observation based on their differences and further proceeds by joining the most similar clusters at each step in an iterative manner (Handorf *et al.*, 2018). The computed HCA models show distinct clusters corresponding to the samples from the control and infected groups among the **Dunnart** and **SWK001** cultivars (**Figure 4.5B**). Treatment-related (infected) and time-related groupings were also formed within each major cluster of the two respective cultivars (**Figure 4.5D**). To better interpret the biochemical differences revealed by PCA and HCA in oat responding to bacterial infection, a supervised modelling method was used (OPLS-DA)

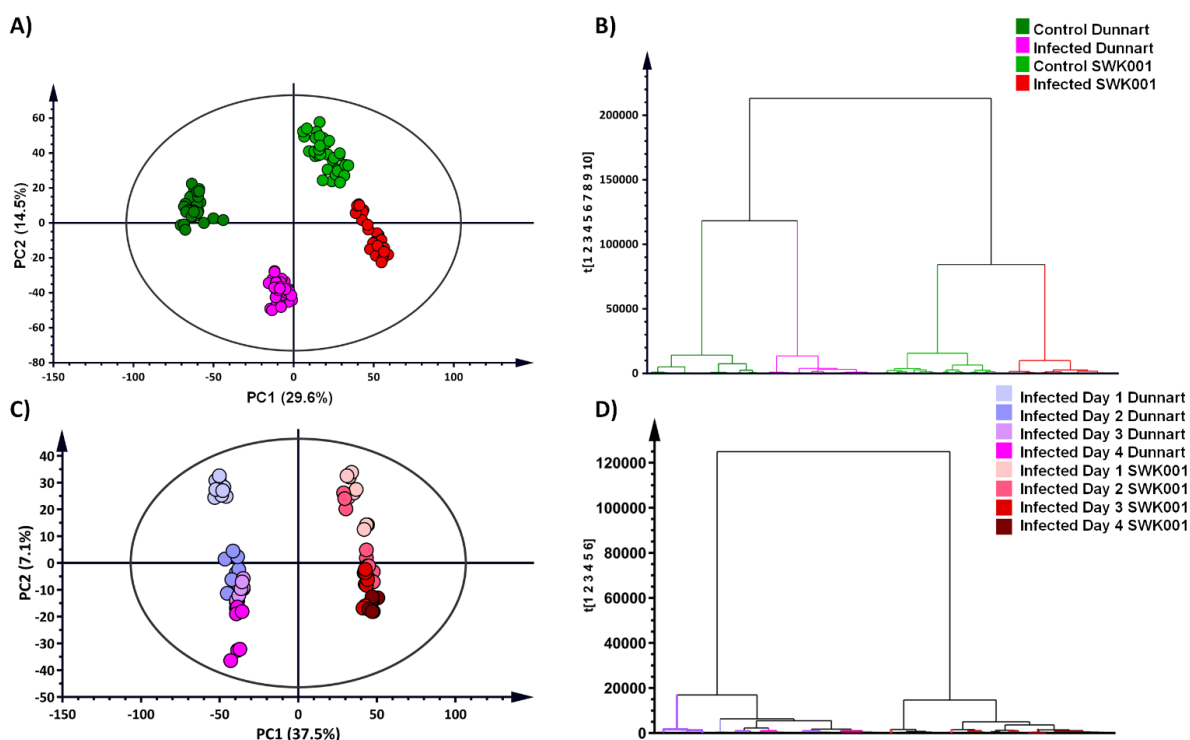


Figure 4.5: Principal component analysis (PCA) of the two infected oat cultivars with the corresponding hierarchical cluster analysis (HCA) dendrograms. PCA scores plots indicate the clustering and general grouping among the infected and control groups for the different cultivars (Dunnart & SWK001) analysed in ESI (-) mode. (A) PCA scores plots of all the samples showing the infected and control groups for Dunnart and SWK001. (C) PCA scores plot of all the infected samples from 1 d.p.i. to 4 d.p.i. for Dunnart and SWK001. (B, D) HCA dendrograms (corresponding to PCA plots A and C respectively) shows the hierarchical structure of the data indicating that the control and infected groups for Dunnart and SWK001 respectively cluster together and are grouped separately from one another. In (D) the infected groups for Dunnart are clustered separately to the left and the infected groups of SWK001 to the right. The unsupervised modelling tools allowed a comprehensive overview of the data (PCA) and grouped the samples with regard to their treatment-related differences from 1-4 d.p.i. and their natural clustering based on cultivar-dependent variation (HCA).

OPLS-DA was applied as a binary classifier that aids in extracting discriminatory variables underlying differential groups (Trygg *et al.*, 2007; Tugizimana *et al.*, 2013). The supervised method ensures separation in the scores-space between different experimental groups, as illustrated (Figure 4.6A & C). The infected and control groups for the Dunnart cultivar were analysed using OPLS-DA modelling (Figure 4.6A) and shows clear separation in the score space, between the control and infected groups. Figure 4.6B also indicated clear clustering and group separation between the Dunnart and SWK001 infected groups. These supervised models were validated using a range of validation methods as previously described in section 3.3.2. The reliability of the models was tested using cross-validation analysis of variance (CV-ANOVA) where the significant models had p -values of <0.05 . Furthermore, the performance of the OPLS-DA models was evaluated using receiver operating models (ROC) where perfect classification was illustrated as the ROC curve passed through the top left corner, indicating

perfect sensitivity and specificity (**Figure 4.7C**). Finally, permutation tests were performed where the OPLS-DA models were statistically shown to be better than the generated permutation models with an R^2 and Q^2 being higher for the initial OPLS-DA model (**Supplementary Figure S4.3**). The OPLS-DA loadings S-plots (**Figure 4.6C & D**) were used to target and select statistically discriminatory ions among the treatment and control groups of the two cultivars, as well as between the treated groups of **Dunnart** and **SWK001**. OPLS-DA models and their corresponding loadings S-plots were constructed for the control *versus* treated groups, time points and infected **SWK001** *versus* **Dunnart** (18 in total for each cultivar – not shown, but available on request). The statistical significance and discriminability of the potential markers obtained from the S-plots were validated using variable trends (**Figure 4.7A**), dot plots (**Figure 4.7B**) and VIP plots (**Figure 4.7D**). The variable trend plot illustrates how the potential markers discriminate between two groups. Furthermore, each selected variable from the S-plot was evaluated using a dot plot (**Figure 4.7B**) that computes each observation as a unit and subsequently sorts each component into “bins” that represent sub-ranges. Strong discriminating variables show no overlap between the groups (Wilkinson, 1999; Tugizimana *et al.*, 2016) as can be seen in **Figure 4.7B**. The VIP plots illustrate VIP values in the form of a column chart, thereby providing means to assess the importance of the variables in explaining how the X and Y variables correlate to one another (Farrés *et al.*, 2015). S-plot variables with VIP scores >1.0 and no overlap between groups (illustrated by the dot and trends plots) were selected for further investigation (**Table 4.1**).

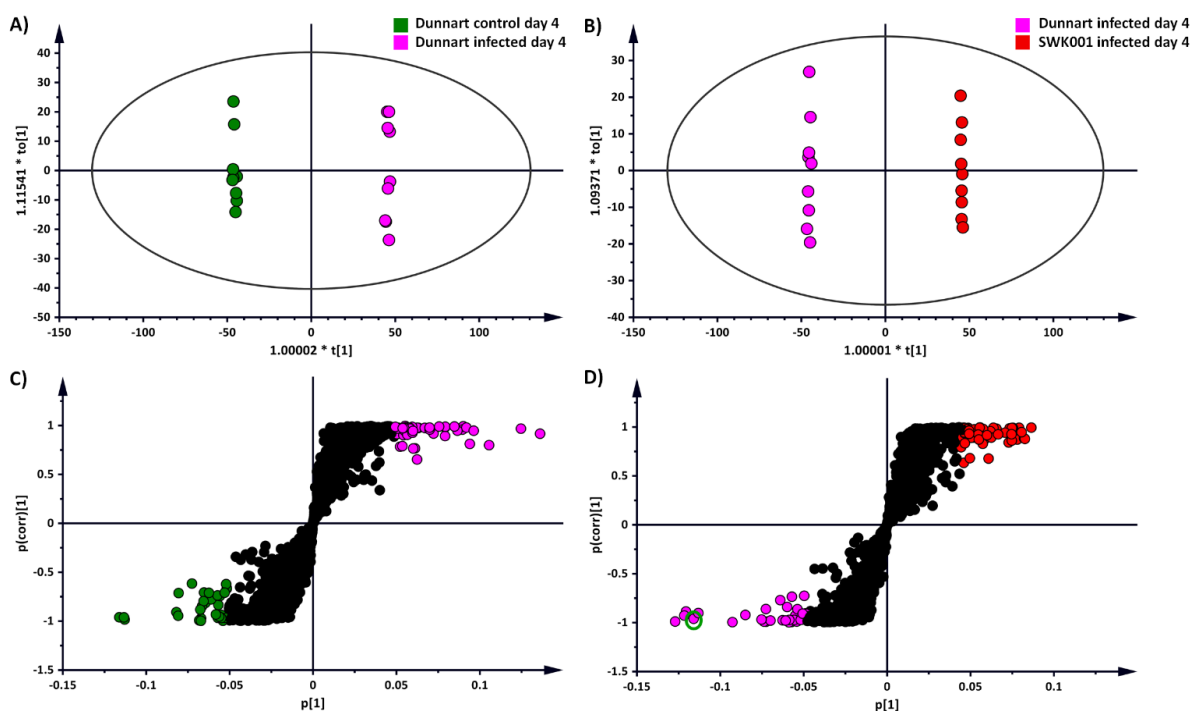


Figure 4.6: An orthogonal projection to latent structures discriminant analysis (OPLS-DA) model of the two infected cultivars: **Dunnart** and **SWK001**. (A) An OPLS-DA scores plot summarising the relationship among different datasets to visualise group clustering between the control and infected **Dunnart** groups at 4 d.p.i.. based on their leaf-extracted metabolic profiles obtained in ESI(-) MS mode ($R^2=0.999$, $Q^2=0.995$, CV-ANOVA p -value= 1.89349×10^{-15}). (B) OPLS-DA scores plot illustrating the relationship among the infected **SWK001** and **Dunnart** crops at 4 d.p.i. based on their leaf-extracted metabolic profiles obtained in ESI(-) MS mode ($R^2=0.999$, $Q^2=0.996$, CV-ANOVA p -value= 1.06365×10^{-14}). (C) The corresponding OPLS-DA loadings S-plot of (A). The pink and green circles indicate the values situated far out ($p[1] > 0.05$, < -0.05 and $p(\text{corr}) > 0.5$, < -0.5) in the S-plot, representing statistically significant ions that are possible discriminatory variables between the control and infected **Dunnart** groups. (D) The corresponding OPLS-DA loadings S-plot of (B). The pink and red circles indicate the values situated far out ($p[1] > 0.05$, < -0.05 and $p(\text{corr}) > 0.5$, < -0.5) in the S-plot, indicating statistically significant ions that are possible discriminatory variables between the infected **SWK001** and **Dunnart** crops. The green circle indicates the selected variable- avenanthramide A for which validation models are shown in Figure 4.7.

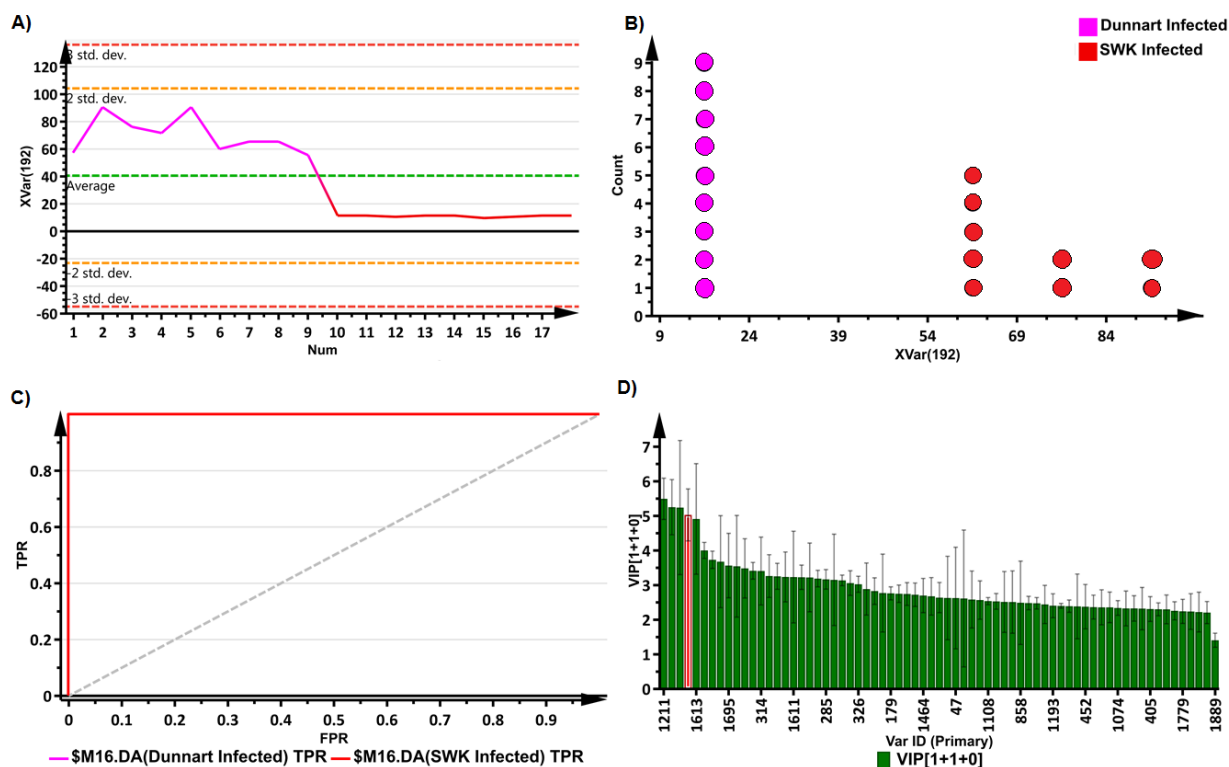


Figure 4.7 S-plot and OPLS-DA representative validation models. (A) Variable trend plot illustrating the selected variable, avenanthramide A, illustrated in red on the VIP plot (D) and circled on the S-plot (Figure 4.6D), that displayed changes among the Dunnart and SWK001 infected groups at 4.d.p.i. (B) Dot plot illustrating strong discrimination between infected groups of the SWK001 and Dunnart cultivars for the selected variable-avenanthramide A as there is no overlap between the groups. (C) Receiver operator characteristic (ROC) plot for the OPLS-DA model (Figure 4.6B) separating the infected groups at 4 d.p.i. The ROC plot is a graphical summary of the performance of a binary classifier. A model with perfect discrimination has a ROC curve with 100% sensitivity and 100% specificity, as the curve passes through the top left corner. (D) A variable importance for the projection (VIP) plot for Figure 4.6B, illustrating the importance of each variable contributing to the discrimination of the two groups.

4.3.4 Metabolic profiling of *Ps-c* induced changes in oat cultivars

For biochemical interpretation of metabolic changes in the leaf tissue of the oat cultivars responding to infection by *Ps-c*, as graphically illustrated by the chemometric models, the statistically selected metabolites from the loadings S-plots were annotated and putatively identified (Table 4.1) and interpreted for their potential biological roles in oat defence against *Ps-c*. The metabolites were annotated as previously described in section 4.2.8, and classified into the following metabolite groups: amino acids, phenolics, phenolic amides, fatty acids, flavonoids, alkaloids, terpenoids, lipids, saponins and plant hormones. Induced changes across these metabolite classes in the Dunnart and SWK001 cultivars were metabolically characterised and showed alterations involved in the plant response to the bacterial pathogen.

Among the triggered alterations, plant hormones were identified; namely jasmonates (jasmonoyl-isoleucine, jasmonic acid-L-valine) and traumatic acid. Jasmonates are produced as a signalling hormone in response to pathogen attack and causes the plant to reconfigure its metabolism to produce potent defensive secondary metabolites (Zhang *et al.*, 2017). This process is facilitated by the jasmonate signalling pathway that leads to the rapid accumulation of jasmonic acid (JA) and its bioactive form jasmonoyl-L-isoleucine, which presented as a discriminatory metabolite for **SWK001** treatment, contrary to the **Dunnart** treatment, where it did not present as discriminatory. These signalling hormones cause transcriptomic and metabolic reconfigurations that aid in plant defence responses (Woldemariam *et al.*, 2014; Bedini *et al.*, 2018). Many different jasmonate precursors and derivatives have been known to exhibit biological activity, including jasmonic-amino acid conjugates (Wasternack & Strnad, 2018), of which two were identified in this study in response to bacterial infection. Traumatic acids are organic compounds that are often referred to as plant growth and development regulators that participate in the regulation of plant metabolism (Pietryczuk *et al.*, 2014). Traumatic acid is generally classified as a wound hormone and has been found to accumulate in large quantities around plant wounded areas (Jabłońska-Trypuć *et al.*, 2016). It has, however, also been identified as a resistance inducing metabolite for barley against *Fusarium* head blight disease (Chamarthi *et al.*, 2019) and its detection in oats due to *Ps-c* infection suggest an additional defensive role. Traumatic acid and JA biosynthesis occurs from the octadecanoic pathway *via* either linoleic acid or linolenic acid (Yan *et al.*, 2013; Jabłońska-Trypuć *et al.*, 2016). These are both 18-carbon unsaturated fatty acids precursors that were also identified among the discriminatory metabolites. The synthesis of these hormones initiates a physiological response in plants to increase the production of defence related compounds like phenolics, flavonoids, alkaloids and terpenes (Yang *et al.*, 2018).

Phenolic acids are produced and accumulate in plant tissues in response to stress and/ or pathogen attack, where they act as protective agents against invading organisms like insects, fungi, nematodes and bacterial pathogens (Bhattacharya *et al.*, 2010). As previously described, phenolic acids are produced by the phenylpropanoid pathway from phenylalanine through deamination, hydroxylation and methylation (Vogt, 2010). A unique group of phenolic acid amides were identified as discriminatory in the treated groups of both **Dunnart** and **SWK001** and were absent in the respective controls. These phytoalexins, known as avenanthramides, are unique to oat crops and consist of an anthranilic acid bound to a hydroxycinnamic acid *via* an amide bond (**Chapter 2, Figure 2.8**). Over 40 different types of avenanthramides have been

identified and classified based on their structure in oat leaves and grains. The most abundant avenanthramides are A-C (Tripathi *et al.*, 2018; Li *et al.*, 2019), which were identified as discriminatory ions/ metabolites for the **Dunnart** treated groups. Avenanthramide L was also identified in both the **Dunnart** and **SWK001** treated groups. The biosynthesis of these compounds is initiated (as described in **Chapter 2, section 2.3.6**) by the synthesis of coumaric acid from phenylalanine, both of which presented as discriminatory metabolites for the treated groups. These compounds are produced in response to the pathogen infection or when oat leaves are treated with various elicitors, hence, avenanthramides are produced by oat crops for its defence (Ren & Wise, 2013). As phytoalexins, these compounds function as both chemical defence and as substrates for the reinforcement of cell walls in physical defence upon exposure to pathogens (Okazaki *et al.*, 2004). The greater abundance and presence of these compounds in **Dunnart**, could have contributed to the defence response and tolerance of the cultivar to the bacteria, in contrast to the **SWK001** cultivar that only presented avenanthramide L as a discriminatory metabolite and showed greater susceptibility in response to the treatment and resulted in severe symptom development.

Alkaloids are a group of nitrogen containing compounds that form part of important secondary metabolites that play a crucial role in plant defence, especially as antimicrobial compounds (Mazid *et al.*, 2011). As secondary metabolites, alkaloids are synthesised from amino acids precursors such as aspartate, lysine, tyrosine and tryptophan. These bioactive compounds have exceptional biological activities mostly attributed to the ability to form hydrogen bonds with proteins, enzymes and receptors, due to the presence of a nitrogen atom (proton accepting) and amine hydrogen group(s) (proton donating) (Parthasarathy *et al.*, 2018; Casciaro *et al.*, 2020). Two alkaloids were identified as discriminatory in the **Dunnart** treated groups that possibly contributed to the antimicrobial activity against *Ps-c*. The first is tubulosine, an isoquinoline alkaloid derived from tyrosine and the second feruloylserotonin (also a hydroxycinnamic acid amide) (Chrzanowska & Rozwadowska, 2004; Li *et al.*, 2018).

Another group of secondary metabolites that greatly contribute to plant defence are terpenoids. Terpenoids are a large group of phytochemicals that exhibit antimicrobial activity. The majority of terpenoids are antimicrobial due to their ability to inhibit two crucial processes necessary for microbial survival, which includes oxidative phosphorylation and oxygen uptake (Guimarães *et al.*, 2019; Mahizan *et al.*, 2019). Here, clerodin, a diterpenoid saponin, was

found as a discriminatory metabolite in leaf extracts from the infected **Dunnart** cultivar, but not in the case of **SWK001**.

Two saponin molecules were also identified as discriminatory between the treated and control groups of both cultivars (avenacoside A and 26-desglucoavenacoside A). As described in **Chapter 3, section 3.3.3**, avenacosides are biologically inactive phytoanticipins that are converted into biologically active 26-desglucoavenacosides by an enzyme known as avenacosidase in response to tissue damage or pathogen attack (Pecio *et al.*, 2013). Here, avenacoside A can be seen as showing a decrease in level from the control to the infected groups, with 26-desglucoavenacoside A increasing in the treated **Dunnart** cultivar. In the context of plant defence, it is of interest that desglucoavenacoside A was not detected among the discriminatory metabolites in **SWK0001**. Thus, a clear response can be seen as the biologically inactive phytoanticipin was converted to its biologically active form upon treatment with *Ps-c*. The major mechanism of action against the pathogen is due to the ability of the compound to complex with sterols in the pathogen membrane and cause disruption in the integrity thereof. This process is thought to result in the formation of transmembrane pores by aggregation of the saponin with the sterol groups resulting in leakage of the cell content and ultimately cell death (Morrissey *et al.*, 2000; Du Fall & Solomon, 2011; Pecio *et al.*, 2013; Moses *et al.*, 2014).

Table 4.1 List of key signatory metabolites extracted and putatively identified from leaves of oat plants treated with *Pseudomonas syringae* pv. *coronafaciens*. These discriminating metabolites were identified based on OPLS-DA S-plots, with a rigorous statistical validation (as explained in the text – **Figure 4.7**). These reported metabolites had VIP scores > 1.0. Increases and decreases are indicated for each treatment where infected (I) and controls (C) were compared for the cultivars **Dunnart- Dun** and **SWK001-SWK**.

Putative Identification	Molecular formula	ESI mode	m/z	Rt (min)	Metabolite Class	Cultivar / Condition			
						Dun (I)	Dun (C)	SWK (I)	SWK (C)
Coumaric acid	C ₉ H ₈ O ₃	Neg	163.0379	3.41	Phenolic acid	●	○	-	-
Phenylalanine	C ₉ H ₁₀ NO ₂	Neg	164.069	1.69	Amino acid	-	-	●	○
Tryptophan	C ₁₁ H ₁₂ N ₂ O ₂	Neg	203.081	2.66	Amino acid	-	-	●	○
Oxododecanoic acid	C ₁₂ H ₂₂ O ₃	Neg	213.147	17.25	Fatty acid	-	-	○	●
Traumatic acid	C ₁₂ H ₂₀ O ₄	Neg	227.126	16.93	Fatty acid	●	○	●	○
Naringenin	C ₁₅ H ₁₂ O ₅	Neg	271.148	8.37	Flavonoid	○	●	-	-
Hydroxylinolenic acid	C ₁₈ H ₃₀ O ₃	Neg	293.211	21.64	Fatty acid	-	-	●	○
Avenanthramide A**	C ₁₆ H ₁₃ NO ₅	Neg	298.069	14.04	Phenolic amides	●	○	-	-
Jasmonic acid-L-valine	C ₁₇ H ₂₇ NO ₄	Neg	308.092	18.36	Amino acid conjugate	-	-	●	○
Avenanthramide C**	C ₁₆ H ₁₃ NO ₆	Neg	314.065	13.06	Phenolic amides	●	○	-	-
Gentisic acid glucoside	C ₁₃ H ₁₆ O ₉	Neg	315.069	1.71	Phenolic acid	-	-	●	○
Jasmonoyl-isoleucine	C ₁₈ H ₂₉ NO ₄	Neg	322.202	20.45	Amino acid conjugate	-	-	●	○
Avenanthramide L	C ₁₈ H ₁₅ NO ₅	Neg	324.085	15.77	Phenolic amides	●	○	●	○
Trihydroxyoctadecadienoic acid	C ₁₈ H ₃₂ O ₅	Neg	327.214	16.68	Fatty acid	○	●	●	○
Avenanthramide B**	C ₁₇ H ₁₅ NO ₆	Neg	328.082	14.51	Phenolic amides	●	○	-	-
Coumaroylquinic acid	C ₁₆ H ₁₈ O ₈	Neg	337.09	3.42	Phenolic acid	●	○	-	-
Feruloylserotonin	C ₂₀ H ₂₀ N ₂ O ₄	Neg	351.127	6.74	Phenolic amide / Alkaloid	●	○	-	-
Rutamarin	C ₂₁ H ₂₄ O ₅	Neg	355.159	1.58	Phenolic acid	-	-	●	○
Sinapaldehyde glucoside	C ₁₇ H ₂₂ O ₉	Neg	369.119	13.63	Phenolic acid	○	●	-	-

Dihydroferulic acid glucuronide	C ₁₆ H ₂₀ O ₁₀	Neg	371.096	5.54	Phenolic acid	-	-	○	●
Syringin	C ₁₇ H ₂₄ O ₉	Neg	371.135	16.02	Phenolic acid	○	●	-	-
Sinapic acid glucose	C ₁₇ H ₂₂ O ₁₀	Neg	385.116	4.52	Phenolic acid	○	●	-	-
Auriculoside	C ₂₂ H ₂₆ O ₁₀	Neg	393.175	12.22	Flavonoid	-	-	○	●
Quercetin dimethyl ether methylbutyrate	C ₂₂ H ₂₂ O ₈	Neg	413.121	16.7	Flavonoid	-	-	●	○
Sophoraflavanone G	C ₂₅ H ₂₈ O ₆	Neg	423.1856	11.83	Flavonoid	○	●	-	-
Vitexin	C ₂₁ H ₂₀ O ₁₀	Neg	431.095	10.98	Flavonoid	○	●	-	-
Clerodin	C ₂₄ H ₃₄ O ₇	Neg	433.234	22.84	Terpenoid	●	○	-	-
Isovolubilin	C ₂₃ H ₂₄ O ₉	Neg	443.133	16.91	Flavonoid	-	-	●	○
Tubulosine	C ₂₉ H ₃₇ N ₃ O ₃	Neg	474.261	21.13	Alkaloid	●	○	-	-
1-Acyl-sn-glycero-3-phosphoglycerol (n-C16:1)	C ₂₂ H ₄₂ O ₉ P	Neg	481.254	22.79	Lipid	●	○	-	-
Isoamoritin	C ₃₁ H ₃₈ O ₆	Neg	505.255	21.51	Flavonoid	●	○	-	-
Formononetin glucoside malonate	C ₂₅ H ₂₃ O ₁₂	Neg	515.247	14.2	Flavonoid	-	-	●	○
Dirhamnosyl-linoleic acid	C ₂₈ H ₄₈ O ₁₁	Neg	559.311	21.9	Fatty acid	●	○	-	-
Isovitexin 2"-O-arabinoside	C ₂₆ H ₂₈ O ₁₄	Pos	563.139	10.33	Flavonoid	○	●	○	●
Vitexin 2"-O-rhamnoside	C ₂₇ H ₃₀ O ₁₄	Neg	577.154	10.75	Flavonoid	○	●	○	●
Acacetin 7-O-rutinoside	C ₂₈ H ₃₂ O ₁₄	Pos	593.149	11.39	Flavonoid	○	●	○	●
Kaempferol rhamnoside galacturonide	C ₂₇ H ₂₈ O ₁₆	Neg	607.132	9.32	Flavonoid	○	●	-	-
Linarin monoacetate	C ₃₀ H ₃₄ O ₁₅	Neg	633.181	9.32	Flavonoid	○	●	-	-
Prenylkaempferol diglucoside	C ₃₂ H ₃₈ O ₁₆	Neg	677.207	14.51	Flavonoid	○	●	-	-
Tricin ether glucopyranoside	C ₃₃ H ₃₆ O ₁₆	Pos	689.193	13.21	Flavonoid	○	●	○	●
Palmitoleic-linoleic glucoside	C ₃₃ H ₃₆ O ₁₆	Neg	723.382	21.85	Fatty acid conjugate	●	○	-	-
26-Desglucoavenacoside A	C ₄₅ H ₇₂ O ₁₈	Neg	945.481	18.45	Steroidal saponin	●	○	-	-
Avenacoside A**	C ₅₁ H ₈₂ O ₂₃	Pos	1063.539	16.58	Steroidal saponin	○	●	○	●

Closed circles (●) and open circles (○) indicate increases and decreases respectively (positively / negatively correlated to the condition) (-) Indicates metabolites that did not present as discriminatory ions in the respective treatments and cultivars.

(**) Metabolite identity was confirmed with an authentic analytical standard – MSI level 2.

As before, data visualisation tools were used to illustrate the magnitude and presence of the respective metabolites in various treatment and control groups for the respective cultivars with heatmap analysis (**Figure 4.8**). Here, the average integrated peak areas of the respective metabolites were used to construct heatmaps using statistical analysis software available on MetaboAnalyst (Chong *et al.*, 2018). The infographic shows clear differences among the cultivars with respect to the treated and non-treated groups. For example, the discriminant features / chemical profiles of the **Dunnart** vs. **SWK001** cultivars becomes obvious and readily offers an explanation for the observed resistant vs. susceptible phenotypes. These profiles could demonstrate to be useful in providing information on the chemical basis of disease resistance in oat against *Ps-c*, and identify metabolic markers associated with resistance or susceptibility traits.

Among the identified metabolites (**Table 4.1**), the differential metabolic profiles based on discriminatory ions present in the hydromethanolic extracts of the treated and control groups were as follows: **Dunnart infected** had 2 flavonoids, 2 phenols, 2 fatty acids, 4 phenolic amides, 2 alkaloids, 1 lipid and 1 saponin (26-desglucoavenacoside A) compared to the **Dunnart control** that showed a metabolic profile containing 9 flavonoids, 2 phenols, 1 fatty acid and 1 saponin (avenacoside A). **SWK001 infected** showed a metabolic profile containing 3 flavonoids, 2 phenols, 4 amino acids and 3 fatty acids. In comparison, the **SWK001 control** showed a metabolic profile containing 5 flavonoids, 1 phenol, 1 fatty acid and 1 saponin (avenacoside A). Based on these differential metabolic profiles, partial overlap and clear differences can be seen among the cultivars as well as the treated and control groups in the form of a Venn diagram (**Figure 4.9**).

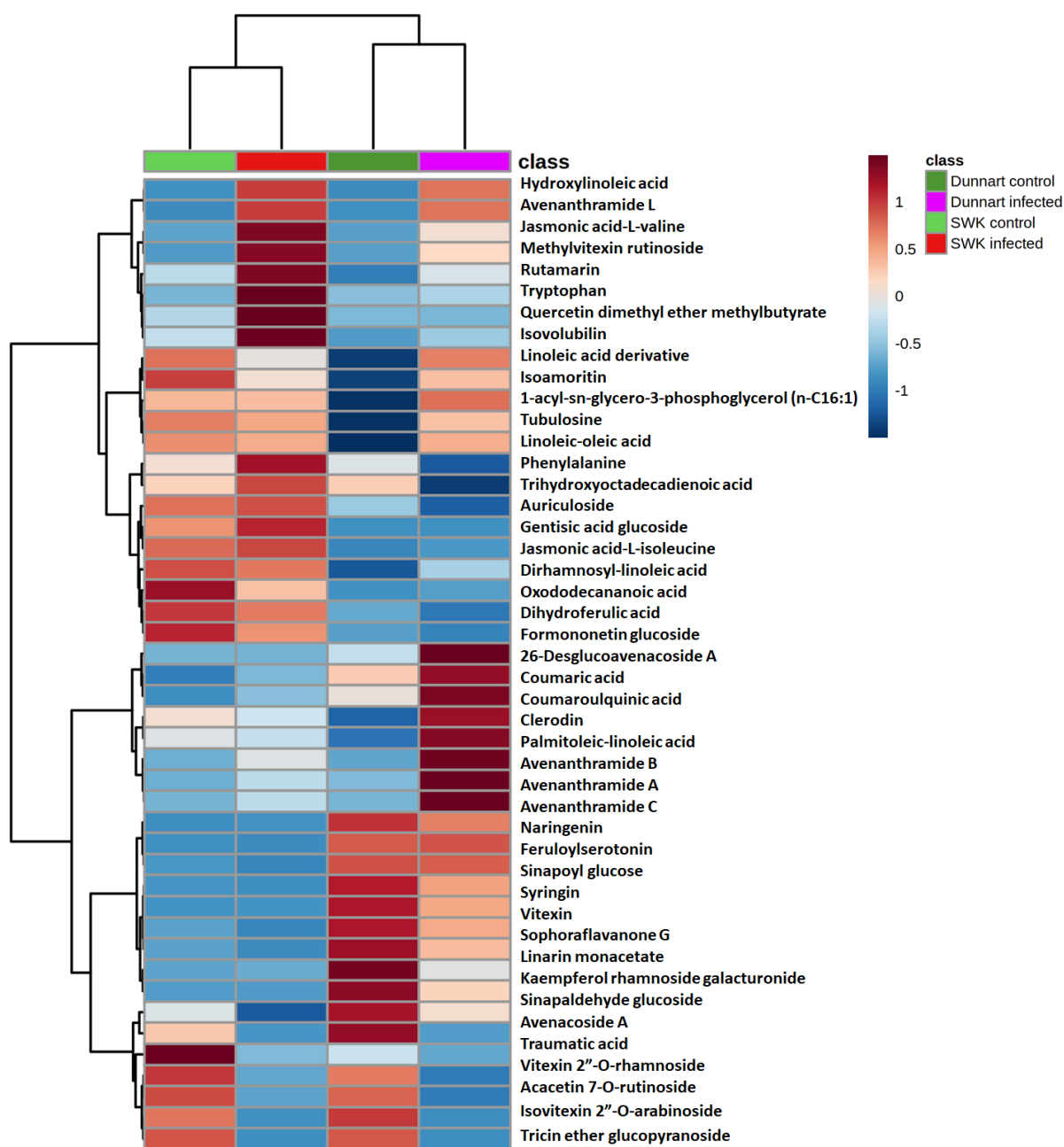


Figure 4.8 Heatmap analysis of individual peak intensities of the putatively identified metabolites from oat leaves treated with *Ps-c*. The map was constructed (using the Pearson distance and Ward's linkage rule) to illustrate infected and control groups of the two respective cultivars, **Dunnart** & **SWK001**. The mean peak intensities of each annotated metabolite are shown after Pareto-scaling of the data. Values higher than the averages are shown in **brown** and lower in **blue**, with each row representing discriminant features and each column representing the respective cultivars and treatment groups.

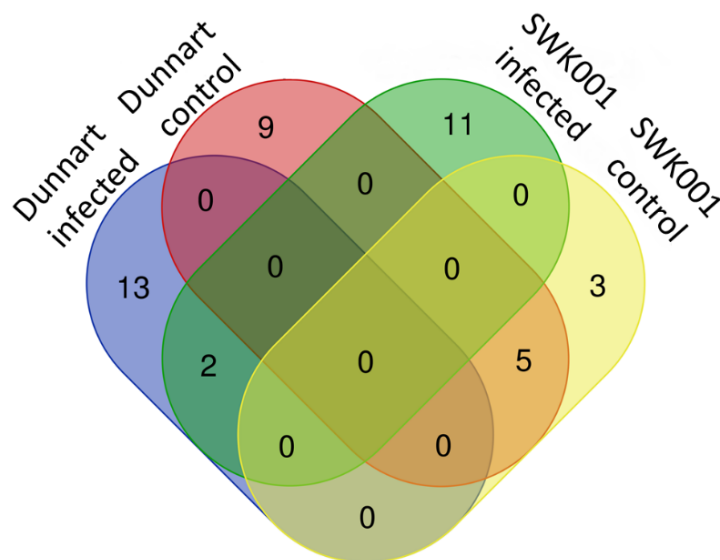


Figure 4.9: Venn diagram displaying the partial overlap and differences of statistically significant variables selected from the OPLS-DA models. Extracts from the infected and control groups of the respective cultivars (**Dunnart** and **SWK001**) are compared. The numerical values in the diagram represents the discriminatory metabolites (**Table 4.1**) that are unique to the respective cultivars or treatments and conversely also shared between the cultivars or treatments.

The **Dunnart** cultivar had a number of metabolites that uniquely presented as discriminatory for the infected group compared to the controls (coumaric acid, traumatic acid, avenanthramide A, B, C and L, coumaroylquinic acid, feruloylserotonin, clerodin, tubulosine, 1-acyl-sn-glycero-3-phosphoglycerol (n-C16:1), isoamoritin, dirhamnosyl-linoleic acid, palmitoleic-linoleic glucoside and 26-desglucoavenacoside A) as illustrated in the heatmap (**Figure 4.8**). These metabolites are thus potential biomarkers for the defence response of this particular cultivar to *Ps-c*. The infected **SWK001** groups showed a number of discriminatory metabolites compared to the controls (phenylalanine, tryptophan, traumatic acid, hydroxylinolenic acid, jasmonic acid-L-valine, gentisic acid glucoside, jasmonoyl-isoleucine, avenanthramide L, trihydroxyoctadecadienoic acid, rutamarin, quercetin dimethyl ether methylbutyrate, formononetin glucoside malonate and isovolubilin), making these metabolites possible metabolic biomarkers for the response of this cultivar to *Ps-c*.

Coumaric acid, as an example, is shown as a discriminatory metabolite for the **Dunnart** infected group. It serves as an entry point into the phenylpropanoid metabolic pathway of secondary

metabolites (**Figure 4.10**) which is further confirmed by **Figure 4.11A & D** (pie chart), illustrating the pathways in which this metabolite is involved and how it is distributed amongst the respective cultivars and treatments as shown in **Figure 4.12** (radar charts). When comparing the metabolites involved in the response to the bacteria, the increasing presence and abundance of avenanthramides from 1-4 d.p.i. among the two cultivars were illustrated using colour-coded PCA scores plots, where **Dunnart** contained avenanthramide A-C and L at an increasing presence from 1-4 d.p.i. in comparison to **SWK001** that contained only avenanthramide L (**Supplementary Figure S4.4**). Based on the distribution and presence of these metabolites, it is clear that the two cultivars had different metabolic alterations in response to *Ps-c*, and that the metabolomics method allowed a rapid and sensitive means of detecting the presence of specific secondary metabolites among the different treated cultivars, making it useful for biomarker discovery related to plant-pathogen interactions.

Defence-related metabolites do not work in isolation, but are rather interconnected to each other in different metabolic pathways. For biochemical interpretation of the post-infection metabolic perturbations in oat plants, metabolic pathway mapping was performed to elucidate the most relevant pathways involved in oat responses to *Ps-c* infection. To further analyse the metabolomic reprogramming induced by *Ps-c* infection, metabolomics pathway analysis (MetPA) was performed (MetaboAnalyst 4.0). This highly sensitive web-based tool is useful in the analysis and visualisation of metabolomic data and can detect subtle changes among different metabolites. As a result, biological pathways can be generated based on these concentration changes, or alternatively from a compound list with known KEGG or HMDB compound IDs (Xia & Wishart, 2010; Chong *et al.*, 2018). The computed metabolic pathways are presented according to significance or pathway impact as shown in **Figure 4.10**. The most significant pathways (displayed on the y-axis) were the phenylpropanoid - and flavonoid pathways, whereas the most impactful pathways (displayed on the x-axis) were the linoleic acid - and (in general) the secondary metabolite biosynthesis pathway.

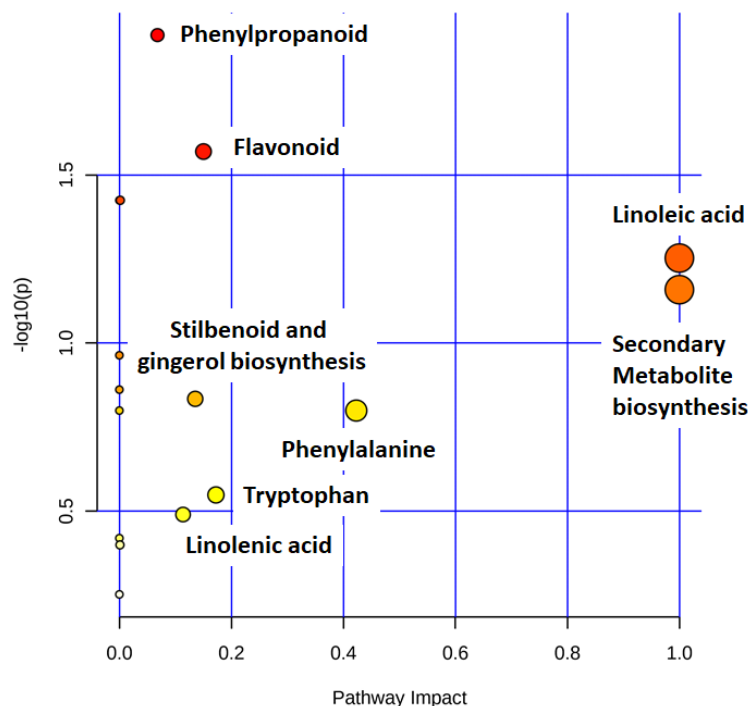


Figure 4.10: Pathway analysis summary of all MetaboAnalyst-computed metabolic pathways displayed according to their significance or pathway impact. The figure illustrates all the matched pathways arranged by p -values (y-axis; pathway enrichment analysis) and the pathway impact values (x-axis; pathway topology analysis). Each node is coloured according to their corresponding p -values, with the node sizes determined according to their impact values. The graph thus illustrates the pathways with high impact: linoleic acid (C18:2, n-6) pathway and the secondary metabolite biosynthesis pathway, furthermore the pathways with high statistical significance were: phenylpropanoid and flavonoid biosynthesis pathways.

In addition to the heatmaps (Figure 4.8), differences with regard to the relative intensities of identified discriminatory metabolites were also explored among the treated and control groups using colour-coded PCA scores plots (Supplementary Figure S4.4), pie charts (Figure 4.11) and radar charts (Figure 4.12). The relative intensities of the different metabolites are illustrated *via* pie charts (Figure 4.11) among the different pathways. The metabolites synthesised *via* the phenylpropanoid - and flavonoid pathways are some of the most widely occurring secondary metabolites that are involved in plant development and defence against biotic and abiotic stresses, such as phenolics, flavonoids, coumarins and lignin (Fraser & Chapple, 2011; Sharma *et al.*, 2019). Both pathways start with the conversion of phenylalanine to p -coumaroyl-CoA and show some overlap as illustrated (*) in Figure 4.11B. The presence and distribution of the phenolic compounds, from both the phenylpropanoid and flavonoid pathways, across the plant kingdom at a cellular, tissue and organ level reiterates the vast biological and biochemical functions important to the survival of plants (Falcone-Ferreira *et al.*, 2012; Deng & Lu, 2017). Phenolics have been known to play important roles in plant-

pathogen interactions as either pre-formed (phytoanticipins) or induced anti-pathogenic molecules (phytoalexins) (Lattanzio *et al.*, 2006). As previously mentioned, linoleic acids (C18:2) are unsaturated fatty acids that are abundant in plant membranes, thus making them important for plant structure and maintaining water permeability and, additionally, are involved in the synthesis of jasmonate, which act as signalling molecules in response to tissue damage caused by pathogens, insects, herbivores or mechanical stress (Wasternack & Song, 2017).

The secondary metabolite biosynthesis pathway (**Figure 4.11D**), shows coumaric acid converted to *p*-coumaroyl-CoA and caffeoyl-CoA converted to feruloyl-CoA, the two precursor metabolites involved in the synthesis of avenanthramides. As illustrated in **Figure 2.8, Chapter 2**, avenanthramide biosynthesis is initialised by the enzymatic synthesis of *p*-coumaric acid from phenylalanine by PAL and C4'H. *p*-Coumaric acid is then transformed into its activated CoA thioester by 4CL, to condense with 5-hydroxyanthranilic acid, catalysed by HHT, to form avenanthramide A. Conversely, the *p*-coumaroyl-CoA is often first converted to *p*-coumaroyl shikimate or quinate, in which case it becomes hydroxylated by *p*-coumaroyl CoA ester 3'-hydroxylase to form caffeoyl-CoA. Subsequently, the caffeoyl-CoA is condensed with 5-hydroxyanthranilic acid in the presence of HHT to form avenanthramide C. Finally, avenanthramide C is methylated by the caffeoyl-CoA O-methyltransferase enzyme to form avenanthramide B (Matsukawa *et al.*, 2002; Yang *et al.*, 2004; Li *et al.*, 2019).

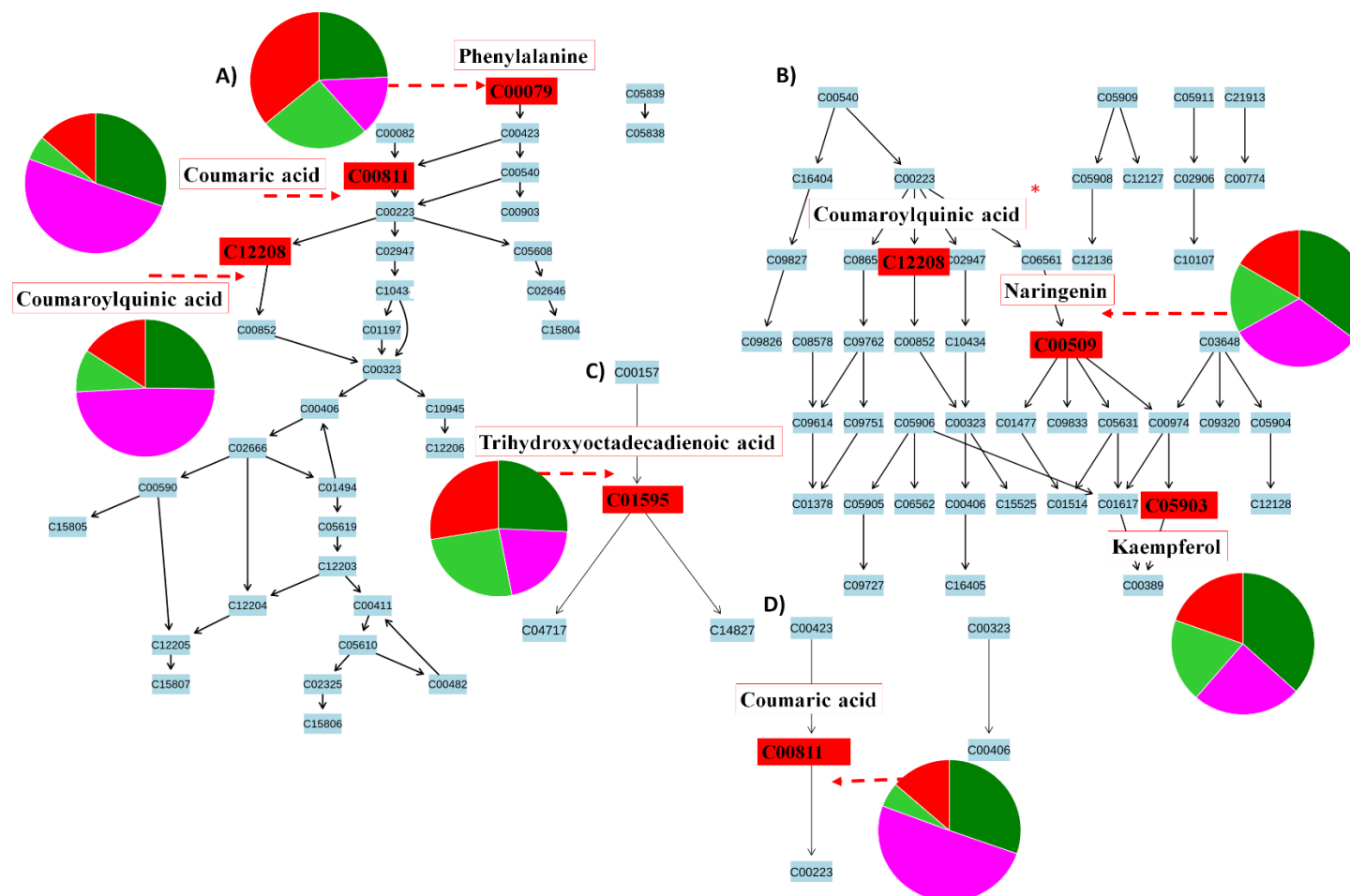


Figure 4.11: Pathways flagged from metabolomics analysis using MetaboAnalyst software. Signatory metabolites involved in each pathway are illustrated in the form of pie charts according to their relative intensities and presence across the different cultivars and treated groups. **(A)** Phenylpropanoid pathway, **(B)** Flavonoid pathway overlapping with the phenylpropanoid pathway (*) and **(C)** The linoleic acid pathway that showed the highest impact after pathway enrichment analysis, along with **(D)** The secondary metabolite biosynthesis pathway. Some limitations in MetaboAnalyst prevented the mapping of all annotated metabolites (**Table 4.1**). The different colours indicate **Dunnart infected**, **Dunnart control**, **SWK001 infected** and **SWK001 control**.

VIP scores plots (**Supplementary Figure S4.5**), derived from partial least squares discriminant analysis (PLS-DA) analysis performed in MetaboAnalyst, indicates the key discriminatory metabolites with a VIP score of >0.5 which are considered significant in discriminating between the *Ps-c* treatment and controls among the two cultivars. Some examples can be seen, as in the case with avenanthramide L (**Figure S4.3**), which presented as a discriminatory feature in the **Dunnart** and **SWK001** treated groups, with **SWK001** showing a greater abundance. The averaged peak intensities of each metabolite were also combined as before to produce radar charts /radial plots (**Figure 4.12**), this time comparatively displaying the metabolic alterations among the respective cultivars in response to bacterial infection of the leaf tissue. In the respective radar plots, a range of metabolites are presented and plotted based on their log-transformed averaged peak intensities. In **Figure 4.12A & B** clear differences and correlations can be seen between **Dunnart** and **SWK001** treated and control groups as well as between **Dunnart** and **SWK001** treated groups (**Figure 4.12C**). Avenanthramides A, B and C, for example, are shown to be least abundant in **SWK001** and most abundant in **Dunnart** (**Figure 4.12C**). These charts are therefore informative in differentiating among the various cultivars and treatments based on the respective discriminatory metabolites.

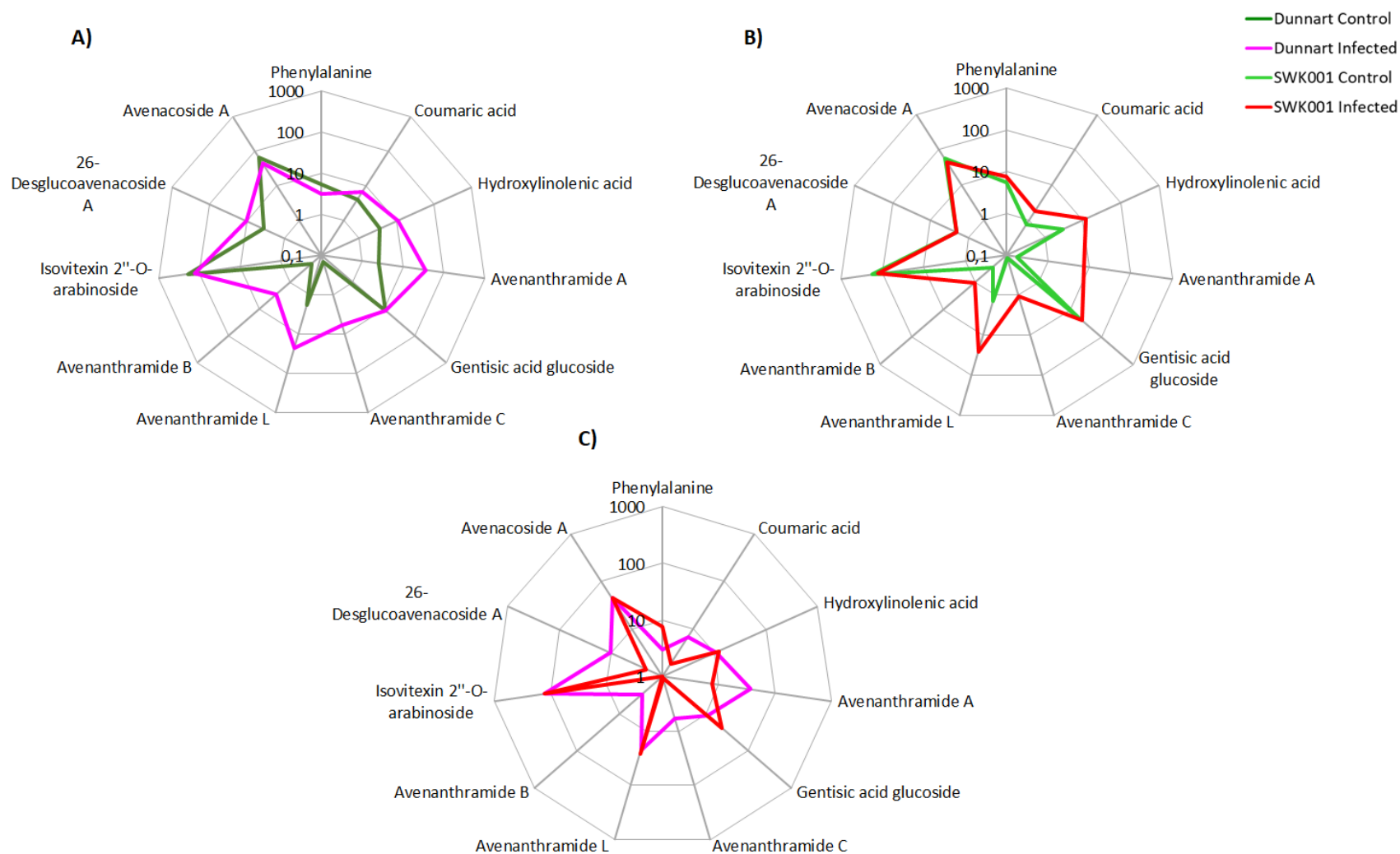


Figure 4.12: Radar charts illustrating relative intensities of selected biomarker metabolites across the different treated and control groups of the oat cultivars. (A) Dunnart infected vs. control, (B) SWK infected vs. control and (C) Dunnart infected vs. SWK infected. The relative peak intensities were averaged and illustrated as log-transformed values.

To summarise, distinct metabolic differences can be seen in the oat cultivars in response to *Ps-c* infection. Based on the phenotypic and metabolic profiles of the respective cultivars, **Dunnart** showed a greater tolerance to *Ps-c*, which can be attributed to the defence metabolites synthesised by this cultivar in an attempt to prevent further pathogen spread and symptom development. **SWK001**, on the other hand, showed severe symptom development that resulted in chlorotic wilted leaves, that could be attributed to the lack of defence metabolites that are both adequate (present in high concentrations) and effective (exhibiting anti-microbial activity), ultimately allowing the pathogen to overcome the plant defence and fully infect the leaves. The Venn diagram displays the partial overlap and differences of the statistically significant variables from the treated and control groups of the two cultivars. The numerical values illustrate the metabolites that were unique to the respective cultivars and treatments (**Figure 4.9**). The **Dunnart** infected group shows the presence of several metabolite classes (phenols, fatty acids, flavonoids, phenolic amides, alkaloids, lipids and saponins) with the three unique phenolic amides (avenanthramide A-C) and 26-desglucoavenacoside A being among the metabolites involved in plant defence for this cultivar. Conversely, the **SWK001** cultivar lacked these phenolic amides and the 26-desglucoavencoside A saponin as discriminant ions. The only overlap illustrated between the defence responses of **SWK001** and **Dunnart** are the presence of traumatic acid and avenanthramide L as discriminatory variables for both these cultivars.

4.4 Conclusion

In the search for new strategies to aid in plant defence against constantly evolving pathogens, a systems biology approach for understanding the biochemical and molecular mechanisms underlying plant immune responses become essential. Oat plants have been greatly underrated, with little research regarding the metabolic response of this cereal to pathogenic threats. Therefore, a comprehensive description of the metabolic mechanisms that underlie oat defence would provide great insight into the resistance and/ or susceptibility events that are involved under biotic stress. In this study, a LC-MS untargeted metabolomics approach was used to obtain a comprehensive understanding of the defensive metabolism of oat plants in response to *Ps-c*. Multivariate data analysis identified signatory metabolites / discriminatory biomarkers from diverse metabolic classes: amino acids, phenolics, phenolic amides, fatty acids, flavonoids, alkaloids, terpenoids, lipids, saponins and plant hormones. Moreover, the study

showed potential metabolic pathways involved in metabolic alterations in response to *Ps-c*, with phenylpropanoid and flavonoid biosynthesis being most significant, and the most impactful pathways being linoleic - and secondary metabolite (avenanthramide) biosynthesis pathways. The secondary metabolite classes that were mentioned have various biological roles that are important in preventing pathogen infections and maintaining the plant under various environmental conditions. Ultimately, an untargeted LC-MS-based metabolomics approach can be used to detect the underlying metabolic alterations and identify metabolic biomarkers that contribute to oat defence response upon inoculation with *Ps-c*. This will greatly contribute to a more holistic view of the oat metabolome under biotic stress and can ultimately be applied in crop improvement and breeding strategies.

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4.5 Supplementary materials

All relevant material referred to, but not included in the main text are included in the following supplementary section. All experimental raw data is available on request at the Department of Biochemistry, University of Johannesburg with permission from Prof. IA Dubery.



Figure S4.1: Typical symptom development of halo blight on Dunnart leaves in response to *Ps-c* infection. Characteristic yellow halo formation around infected leaf tissue, typically associated with halo blight disease.

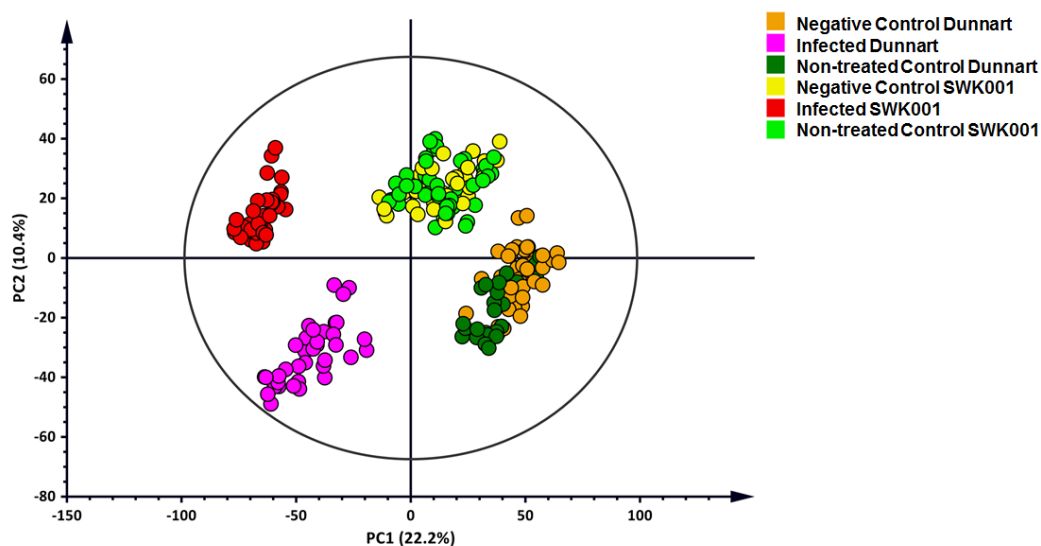


Figure S4.2: Principal component analysis (PCA) of the two infected oat cultivars and the respective control groups. The PCA scores plot of all the samples showing the infected and control groups (non-treated vehicle- and negative control) for Dunnart and SWK001. The non-treated and negative controls clustered together for both cultivars, meaning that there were no significant differences among the controls i.e. the underlying metabolic profiles are similar compared to the infected groups.

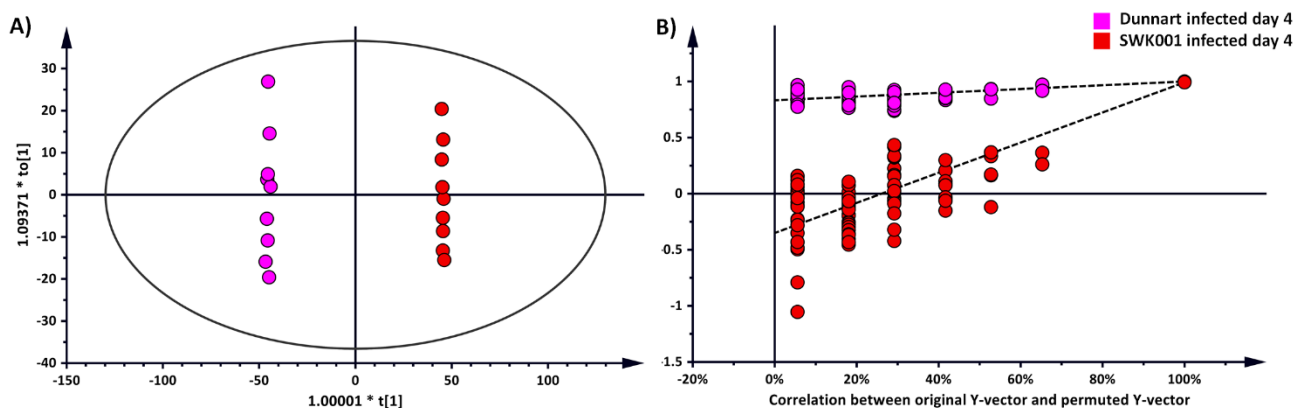


Figure S4.3: An orthogonal projection to latent structures discriminant analysis (OPLS-DA) model of the two infected cultivars, Dunnart and SWK001. (A) OPLS-DA scores plot illustrating the relationship among the infected SWK001 and Dunnart cultivars at 4 d.p.i. based on their leaf-extracted metabolic profiles obtained in ESI (-) mode ($R^2=0.999$, $Q^2=0.996$, CV-ANOVA p -value= 1.06365×10^{-14}). (B) Permutation test plot ($n = 100$) for the OPLS-DA model (A) was used to validate the predictive capability.

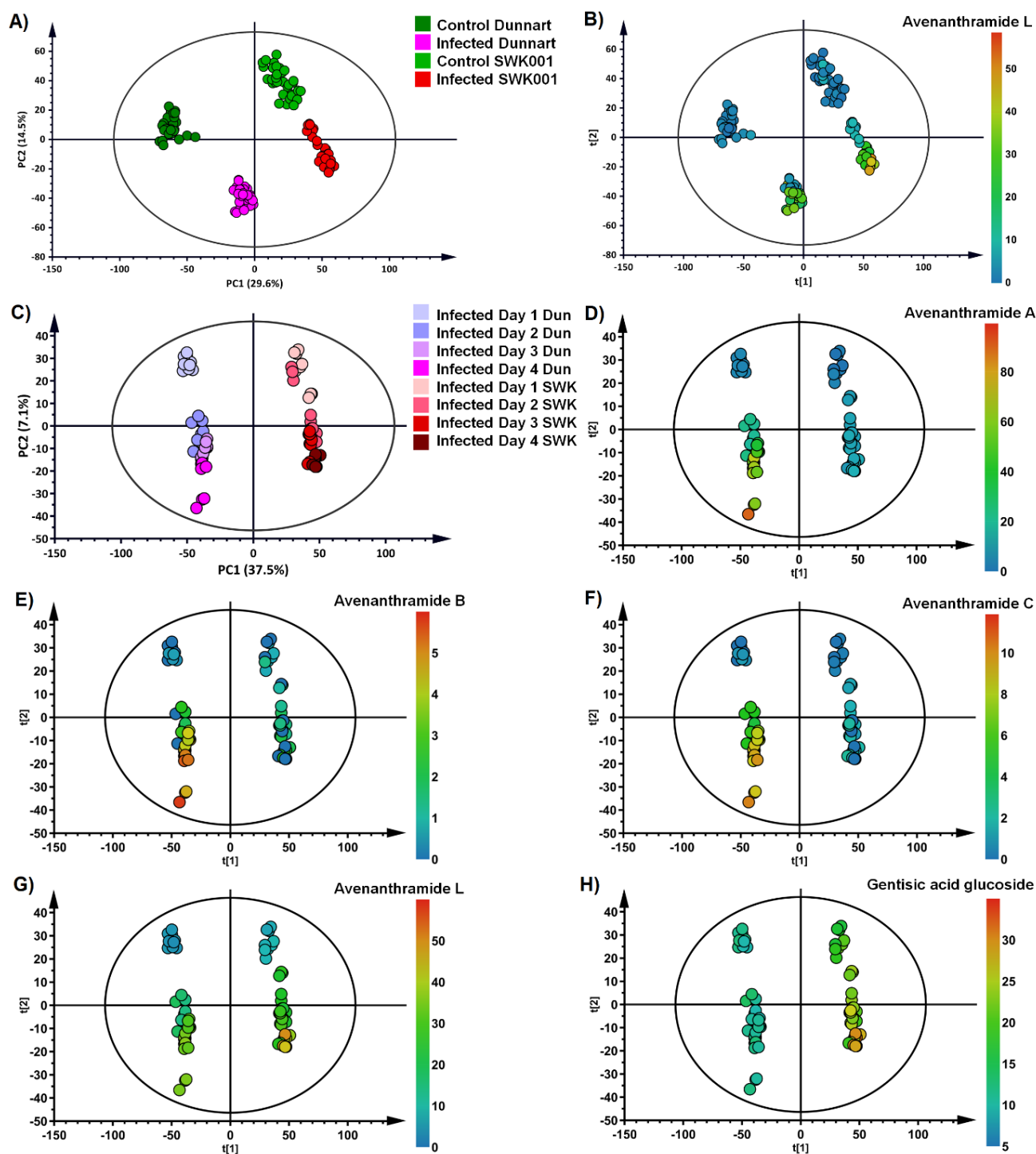


Figure S4.4: Colour-coded PCA score plots showing the presence and increasing abundance of discriminatory ions in the respective treated cultivars, Dunnart & SWK001, (red- high abundance, blue- low abundance). (A) PCA scores plot of both cultivars showing control and infected groups, (B) Avenanthramide L (absent in control groups and present in infected groups), (C) PCA scores plot showing the infected groups for both cultivars from 1 d.p.i. to 4 d.p.i. (D) Avenanthramide A, (E) Avenanthramide B, (F) Avenanthramide C, (G) Avenanthramide L and (H) Gentisic acid glucoside.

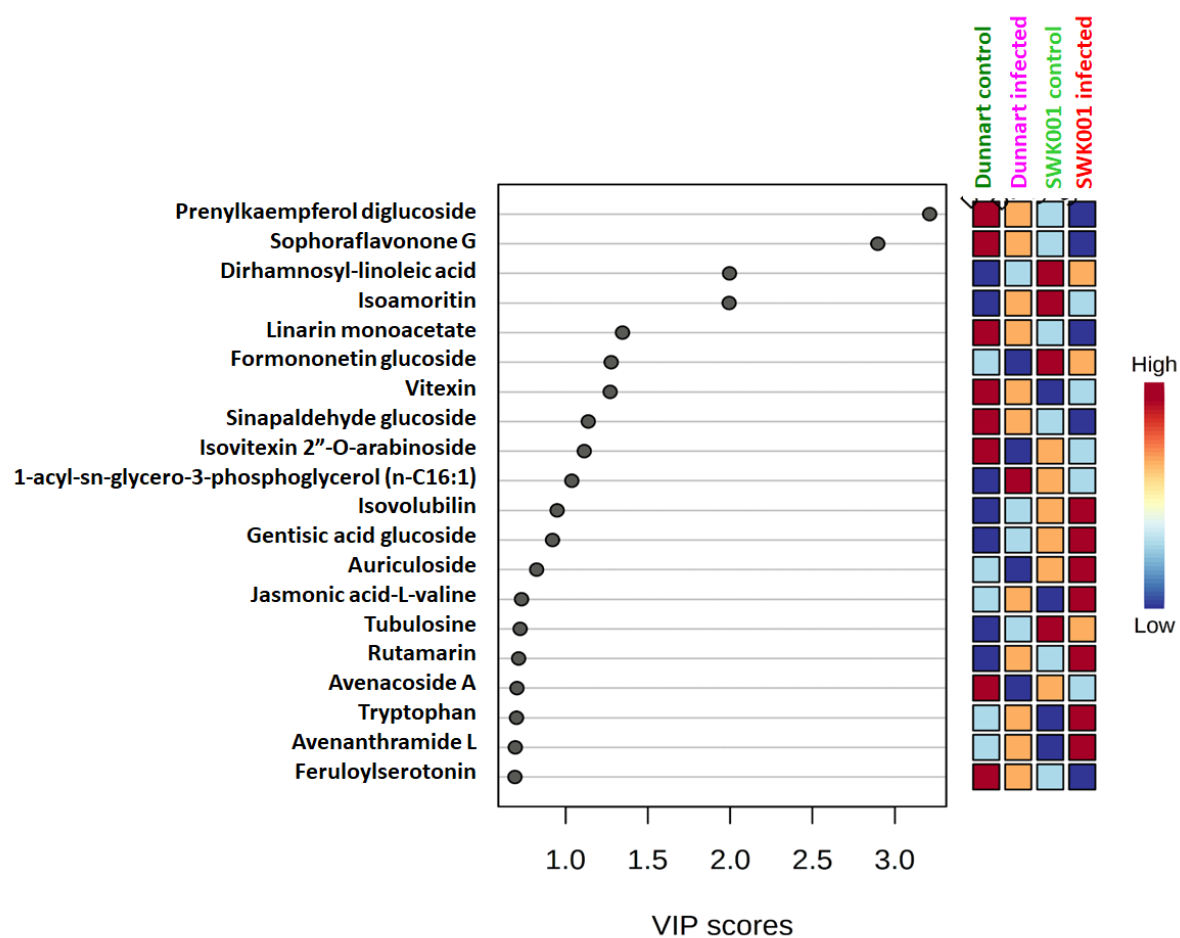


Figure S4.5: Variable importance in projection (VIP) scores generated from partial least squares discriminant analysis (PLS-DA). Indicated are the top 15 discriminating ions in oat leaves from the respective cultivars **Dunnart** & **SWK001** for their treated and control groups. Metabolites with a VIP score ≥ 0.5 were considered to be significant in the discrimination between the cultivars.

4.6 References

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Chapter 5

General conclusion and future prospects

Metabolomics is the comprehensive analysis of all metabolites present within a biological system. The interrogation and analysis of the metabolome thus provides a holistic signature of the physiological state and biochemical processes that occur in a biological system under specific conditions. In this study, metabolomics tools and approaches were applied to unravel differential metabolic profiles of various oat cultivars and to identify signatory biomarkers for cultivar identification as well as oat response to bacterial infection. In this regard, metabolomics has been widely applied in crop plant sciences and has shown great progress in understanding how the phenotype links to the metabolome and, by extension, elucidate the active role of metabolites under normal and stress conditions.

Metabolomics has the potential to provide new approaches to plant breeders for cultivar identification and evaluation of agronomic traits that could result in crop improvement based on chemical composition. These improvements may be nutritional, functional or in the resistance of the crop species to numerous factors. As such, an untargeted LC-MS-based metabolomics approach was applied to interrogate the metabolomes of five oat cultivars. This multidisciplinary approach allowed the elucidation and characterisation of differential metabolic profiles that define natural variation among the respective oat metabolomes. The results further indicated that discrimination among the cultivars not only depended on the presence or absence of specific metabolites, but also on the relative intensities or ratios across the cultivars. Significant biomarkers included diverse classes of metabolites such as carboxylic acids, amino acids, fatty acids, phenolic compounds, flavonoids, steroidal saponin (avenacoside A) and a triterpenoid saponin (avenacin A-1), all of which proved to be discriminating factors across the different cultivars. Metabolites such as hydroxyoctadecatrienoic acid, sinapic acid glucoside, an oxalate derivative and isoquercetin in leaves, and trihydroxyoctadecadienoic acid in roots, were found to be uniquely discriminant

for the **Pallinup** cultivar. Another example was that of **Overberg** which had two uncommon flavonoids that presented as discriminatory features, namely xeractinol and neocarlinoside. The secondary metabolite classes identified here have various biological roles that are important in plant growth and development, preventing pathogen infections and maintaining the plant under various environmental conditions.

Metabolomic alterations in plants upon exposure to stress conditions are dependent on the nature of the stimuli; thus, they have different significance and are expected to variably correlate to stress tolerance. Stress in plants often disrupts metabolic homeostasis and causes the re-adjustment of metabolic pathways. Metabolomics could, thus, elucidate the stress biology in plants by identifying compounds involved in metabolic-related stress responses. In terms of profiling oat response to *Pseudomonas syringae* pv. *coronafaciens* (*Ps-c*), signatory biomarkers were identified as amino acids, fatty acids/ lipids, phenolics (phenolic acid amides and flavonoids), alkaloids, terpenoids, saponins and plant hormones. This suggests that oat defence against pathogen attack involves an integrated response, utilising both primary and secondary metabolism, to support defensive strategies. Moreover, the study showed potential metabolic pathways involved in the response to *Ps-c*, with phenylpropanoid and flavonoid biosynthesis being the most significant, while the most impactful pathways were the linoleic and secondary metabolite (avenanthramide) biosynthesis pathways. A clear chemical defence response could also be seen, where a biologically inactive phytoanticipin (avenacoside A) was converted to the biologically active form (26-desglucoavenacoside A) upon treatment with *Ps-c*. The obtained results support the view that plant defence is based on a multi-layered, complex and highly regulated immune system that involves sensing danger signals and integration of this information to produce appropriate responses to pathogenic challenges.

This study, to the best of our knowledge, is the first to metabolically profile oat cultivars based on their leaves and roots, and provides insight into the metabolic alterations that occur when oat crops are exposed to *Ps-c* inoculation. Among the observations, some interesting correlations can be made with regard to the metabolic profiles of the cultivars based on their natural variation in comparison to that in response to *Ps-c* exposure. As an example, when comparing the **Dunnart** and **SWK001** under normal conditions, the cultivars had unique metabolic profiles and also showed some overlap. Avenacoside A presented as discriminatory molecule for both cultivars but showed a greater abundance in **Dunnart** (**Chapter 3**). After exposure to *Ps-c*, an increasing amount of 26-desglucoavencoside A was observed and

identified as discriminant for the **Dunnart** cultivar (**Chapter 4**). Conversely, no significant conversion was observed in the case of the **SWK001** cultivar and was possibly due to the fact that **Dunnart** had a greater abundance of the avenacoside A initially. Therefore, the metabolic profiles of the cultivars prior to stress exposure, could possibly aid in predicting the ability of the plant to react and produce a successful defensive response.

The untargeted LC-MS-based metabolomics approach proved to be useful in the discrimination between the respective cultivars (metabo-phenotyping), and to detect and characterise underlying metabolic alterations of the host response to inoculation with *Ps-c*. Although the developed method provided a means of distinguishing the respective cultivars and profiling defence-related responses to bacterial inoculation, much remains to be investigated. Metabolomics is a field poised with potential, able to radically reform our understanding of biology and be extended to applications such as the plant – and environmental sciences that include plant-pathogen interactions. In the future, the integration of metabolomics with marker-assisted selection should be considered as an alternative in plant breeding to provide greater coverage and confidence in the discrimination as well as identification of cultivars and favourable traits. Moreover, with constant innovations and improvements of technological and analytic platforms, a faster and more cost-effective metabolomics approach can be expected in the future, which can provide a more holistic understanding of the plant metabolome under growth, development and stress-induced conditions. Ultimately, this work reiterates the use of metabolomics platforms for crop improvement, which in turn will contribute to global food security by aiding in the development of crops with high yield and improved stress tolerance. Additionally, it offers a means of speeding up plant breeding processes that will greatly contribute to crop improvement.

