School of Molecular & Life Sciences

Proteome analysis of *Phytophthora cinnamomi,* the causal agent of Dieback.

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I. Declaration

To the best of my knowledge and belief, this thesis contains no material previously published by another person except where due acknowledgement has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Christina Eleni Andronis

Submitted: 22/08/2022

II. Acknowledgements

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IV. Thesis abstract

This thesis presents a biochemical snapshot of the plant pathogen *Phytophthora cinnamomi. P. cinnamomi* is a root rotting pathogen that causes dieback disease in a variety of native vegetation and several horticultural crops including avocado, macadamia, pineapple and stone fruits. The biochemistry of this organism is not well understood, hence this thesis details in-depth research that aims to better understand the pathogen that can subsequently be used for sustainable methods of management.

Chapter 1 firstly describes the profile of the pathogen from a biological perspective. This includes what is currently known about the life cycle and general pathogenicity of *P. cinnamomi.* This precedes a detailed literature review of the application of proteomic pipelines to general phytopathogenic oomycete research and includes an up-to-date description of the biochemical knowledge of *P. cinnamomi.* This chapter concludes with the gaps in knowledge and aims of this thesis. Chapter 2 describes the general methods and materials used throughout this thesis.

The remainder of this thesis consists of functional research into the biochemistry and pathogenicity of *P. cinnamomi.* Chapter 3 is comprised of published material (Andronis et al. 2021), where mass spectra were used to correct errors in the draft genome and annotate new unsuspected gene models. Using a proteogenomic pipeline, 3% of the detectable proteome was altered to generate an updated genome annotation. Chapter 4 is also comprised of a publication currently under review in the Journal of Proteomics with revisions (Andronis et al. 2022), which defines a detailed proteomic analysis of *P. cinnamomi* sub-proteomes with emphasis on pathogenicity and virulence. Here, I propose a model of metabolic differentiation between cell types and provide a profile of virulence molecules released by the pathogen that have implications on its ability to successfully infect a susceptible host.

The research aim of Chapter 5 was to explore the mode of action of the chemical phosphite on the pathogen and in the plant using a model organism. The mode of action has not been previously confirmed in the literature and this research suggests new and informative biochemical data inferring the multi-modal action of phosphite. Here a model describing the direct and indirect mechanisms of phosphite is presented.

In Chapter 6, the significant outcomes of this research are summarised to demonstrate the overall contribution to *Phytophthora* biology and offers a future perspective and direction for subsequent research.

V. List of abbreviations

MS	Mass spectrometry
LC	Liquid chromatography
MS1	First mass spectrometry analyser
MS2	Second mass spectrometry analyser
1D SDS-PAGE	One dimensional sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
2D SDS-PAGE	Two-dimensional sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
iTRAQ	Isobaric tags for relative and absolute quantification
TMT	Tandem mass tags
PTM	Post-translational modification
R	Resistance gene
Avr	Avirulence gene
TOF	Time of flight
CRN	Crinkler
PAMP	Pathogen-associated molecular pattern
GO	Gene ontology
ROS	Reactive oxygen species
PFAM	Protein family
RP-HPLC	Reverse phase high-pressure liquid chromatography
CONF	Confidence
FDR	False discovery rate
CDS	Coding DNA sequence
GAPDH	Glyceraldehyde phosphate dehydrogenase
JGI	Joint Genome Institute
ORF	Open reading frame
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	KEGG orthologues
EC	Enzyme code

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Chapter 1- Introduction

1.1 AN INTRODUCTION TO PROTEOMICS

The biochemical makeup of any organism provides insight into key factors regarding their roles in survival and relationships to other organisms. These factors can be explored using multi-omic approaches, which can reflect their genomic, transcriptomic, metabolomic and proteomic composition. Tools and technologies designed around elucidating these components enable us to better understand how and why organisms behave and respond in certain ways. This drives to progression of formulating new innovative management strategies that are applicable across many fields from human disease, to crop production.

Proteomics is a powerful tool that enables researchers to take a snapshot of the protein content of an organism in any particular state which informs directly on function. The term 'proteomics' was coined in 1996 to denote the protein products of the genome (Wilkins et al., 1996). The proteome of an organism is the collection of proteins that are found in an organism in a particular state concerning localisation, treatments and interactions at a particular time (Aslam et al., 2017). Mass fingerprinting is the most commonly used method for identifying proteins in a biological sample. In the 1980s, the development of electrospray ionisation and matrix-assisted laser desorption/ionisation overcame challenges previously associated with ion generation in analytical chemistry (Fenn et al., 1989; Dey et al., 1995). This revolutionised the use of mass spectrometry (MS) based proteomics and since has facilitated the biochemical characterisation of countless organisms.

Proteomics can be used to identify, quantify and monitor single proteins in biological samples. Common research focuses typically aim to identify and quantify cell-type specific proteins to gain an understanding of the biochemical processes involved (Shepherd et al., 2003; Resjö et al., 2014; Pang et al., 2016). Typical proteomic pipelines begin with optimised protein extraction of the biological sample followed by proteolytic digestion into peptides (Figures 1a & b). Methods of separation can be implemented at this stage to gain depth into the proteome (Figure 1c). Separated

peptides are subsequently loaded onto a column and eluted into the MS by liquid chromatography (LC) (Figure 1d) where they are ionised and subsequently detected by the first MS analyser (MS1). This produces a set of mass spectra of the detected peptide ions (Figure 1e). Selected ionised peptides are then fragmented and pass through the second MS analyser (MS2), where the fragmented peptide ions are detected (Figure 1f). This produces a second set of mass spectra correlating to the fragmented peptides (Figure 1g). The mass spectra are then matched to an annotated database of the organism, and depending on the experimental design, can be used for protein identification, mapping, quantification or single molecule monitoring.

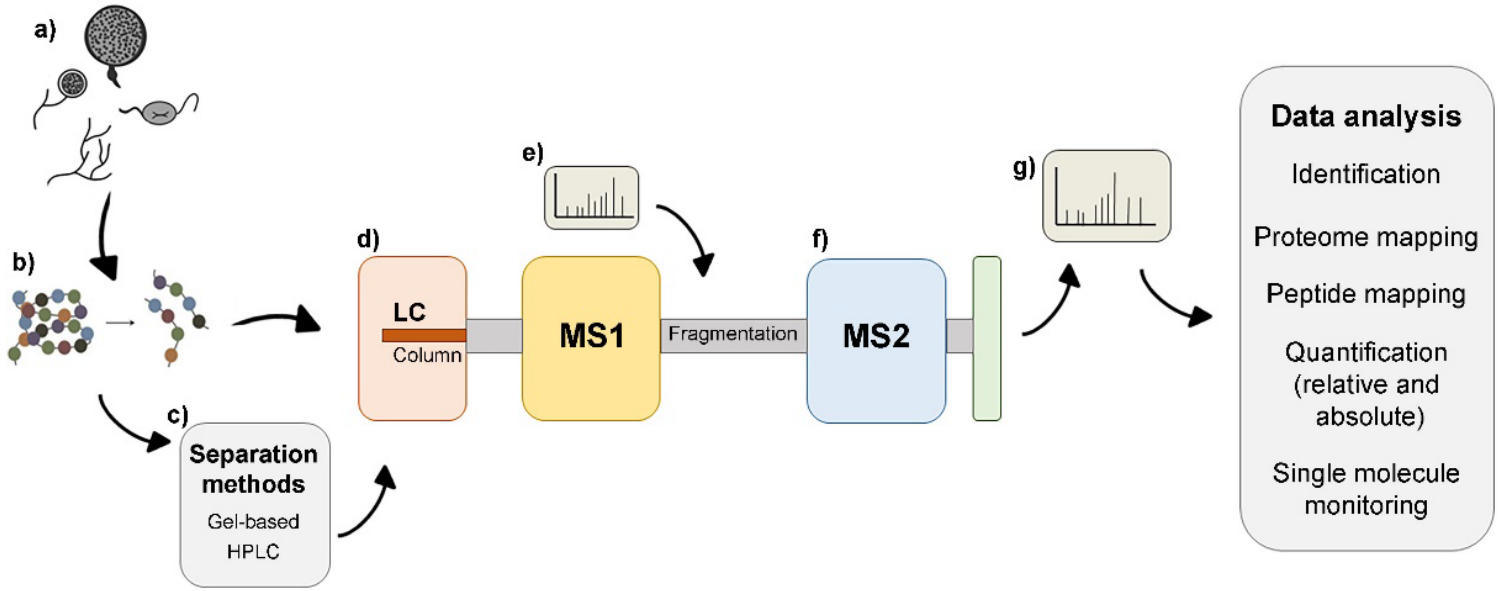

Figure 1. A typical proteomics pipeline. Biological sample of interest **(a)** whose proteins are extracted and subsequently digested into peptides **(b)**. These may or may not require separation **(c)** before peptides are loaded into a column **(d)** and injected into the MS. The peptides are ionised and detected by MS1 **(e)**. Peptides that are selected for MS2 are fragmented and analysed by MS2 **(f)**. The resulting mass spectra are used to match a database **(g)** and subsequently used for various types of data analyses.

Separation overcomes limitations surrounding low abundant proteins that would otherwise be masked during MS detection. One and two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D SDS-PAGE and 2D SDS-PAGE) separation methods are traditionally used to gain depth of proteomes and can be used to investigate both oomycete and plant proteomes (Ebstrup et al., 2005; Zhang et al., 2011; Hosseini et al., 2015). 1D SDS-PAGE was developed in the 1930s, where it was used to separate serum proteins based on their mass (Tiselius, 1937). Whist sufficient for the separation of simple protein mixtures, this method was found to be inadequate for the separation of complex proteomes. 2D SDS-PAGE was developed to overcome these challenges in resolving complex mixtures using a gel-based method by mass and isoelectric point (Smithies and Poulik, 1956). Optimal separation results in deeper proteome coverage as proteins with similar masses are often not well distinguished and are masked by the more abundant proteins. Therefore these lower abundant proteins will not be identified due to the limitations of mass analysers (Matt et al., 2008). Current advances in analytical chromatography have vastly improved the separation of complex protein mixtures and ultimately gained a depth of the detectable proteome by mass spectrometry. These overcome some of the limitations of gel-based separation methods including those around size, protein abundance and hydrophobicity. Whilst gel-based methods have provided valuable information on protein content in different sub-proteomes, chromatographic-based separation allows better dissection of complex protein composition (Savidor et al., 2008; Andronis et al., 2021). Chromatographic separation approaches reduce the complexity of protein samples by fractionating mixtures based on factors such as net surface, charge and hydrophobicity (Fournier et al., 2007; Yang et al., 2012).

Proteomics is highly reliant on genomic data for identification where mass spectra are matched to the genome annotation. This highlights the importance of high-quality genome annotations to facilitate accurate characterisation of protein content and function. Dissecting the proteome by enrichment of sub-proteomes of cell types and cellular compartments allows for a greater diversity of protein detection and validation of a higher proportion of the gene annotation. Protein data can also be used

to aid in the annotation of proteins in related species by homology, building on the repertoire of oomycete networks and their related ontologies (Bringans et al., 2009).

Experimental-based approaches such as subcellular enrichment facilitate the identification and localisation of gene products thereby uncovering useful information such as the regulation of biochemical systems (Grenville-Briggs et al., 2010; Meijer et al., 2014; Hosseini et al., 2015). In addition to qualitative proteomics within and between biological samples, quantitative proteomics can be used to study the differential abundance of proteins, creating a more detailed assessment of these complex systems. Common quantitative comparisons include different cell stages, tissue types and time points. Both labelled and label-free approaches can be used to quantify proteins between different samples (Table 1) (Patel et al., 2009; Wilm, 2009). Most commonly used chemical labels are isobaric tags for relative and absolute quantification (iTRAQ) or tandem mass tags (TMT) (Pichler et al., 2010; Casey et al., 2017). Such tags label tryptic peptides at their N-terminus and lysine residues and allow for the detection and quantification of differentially abundant proteins in a multiplex manner.

Table 1. Comparison between label-free and labelled shotgun proteomics for protein quantification.

	Advantages	Disadvantages
Label-free quantification	No specialised preparation Lower total protein amount required Fractionation not necessary Instrument time < 3 hours	Lower reproducibility Lack of peptide normalisation (performed by total protein amount). No multiplexing
Labelled quantification (iTRAQ, TMT, SILAC)	Lower variability of quantification Multiplexing Reduced technical variability	A higher protein amount required Required fractionation Instrument time >24 hours Costly labels

Post-translational modifications (PTM) can also be identified using proteomics. PTMs modulate the activity of proteins by changing their properties either by the addition

of a modifying group proteolytic cleavage. PTMs can inform on activity state, turnover, localisation and interactions with other proteins.

Proteomic tools alongside genomic data have facilitated the understanding of the biology of important pathogenic microbes, particularly unravelling their virulence, interactions and responses to the environment, and their symbiotic relationship to other organisms.

1.2 Proteomics as a tool for genome annotation

Proteomics has traditionally been used for profiling and comparative analysis of protein abundance. However, alternative use of spectral data has brought about proteomic-assisted genome annotation. With continually increasing mass spectrometry sensitivity and accuracy, the profiles of proteomes are achieved at a greater depth and more peptides are being identified for comprehensive gene validation. Similarly, more high-quality genome sequences are being produced. Discrepancies of correlation between transcripts and proteins have provided a unique opportunity to use mass spectra to manually curate genes. Termed ‘proteogenomics’, this pipeline provides a complementary tool to other gene calling methods such as *in silico* prediction and RNAseq.

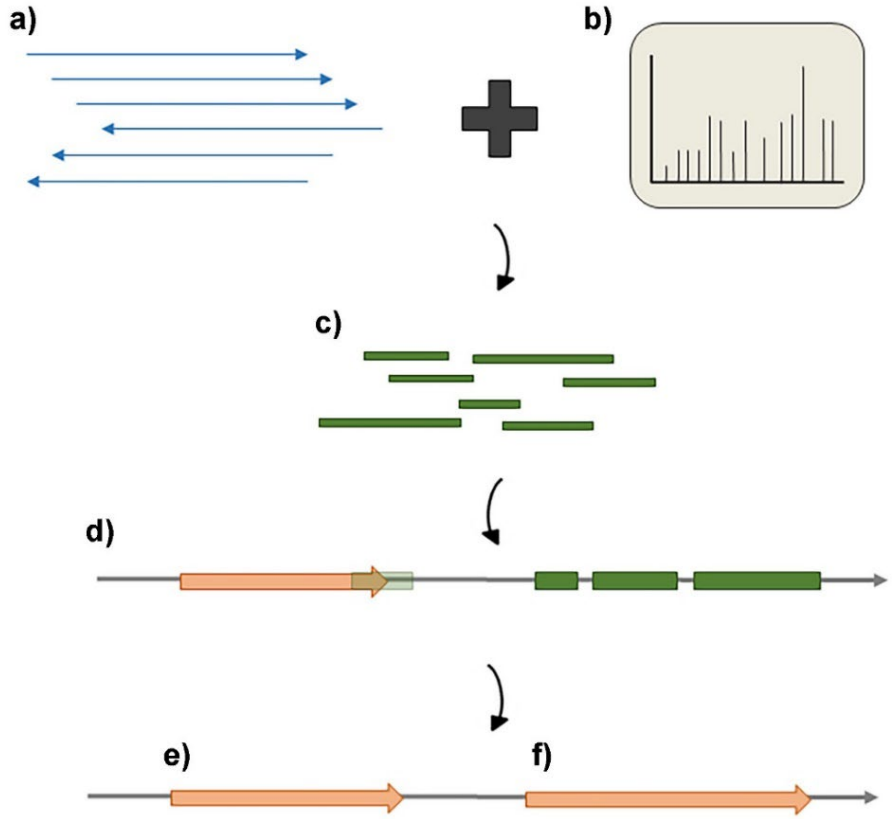

Figure 2. A simplified proteogenomic pipeline for using mass spectra to correct and annotate new genes. a) 6-frame translated genome. b) Mass spectra obtained from organism. c) Match mass spectra to the 6-frame translation to produce 6-frame peptides. d) Map these 6-frame peptides to their genomic location. e) Manually alter of curate new gene models.

Proteogenomics has been used to improve the genome annotation and curate previously unsuspected genes in several organisms including human, mosquito, *Toxoplasma gondii, Arabidopsis thaliana, Anopheles gambiae, Plasmodium falciparum* and *Parastagonospora nodorum* (Lasonder et al., 2002; Desiere et al., 2005; Kalume et al., 2005; Castellana et al., 2008; Xia et al., 2008; Bringans et al., 2009; Imrie et al., 2019). Firstly, a 6-frame translation of the genome is generated (Figure 2a), which is subsequently used as a database to match mass spectra (Figure 2b) resulting in 6-frame peptides (Figure 2c). These peptides are mapped to their genomic location alongside the currently annotated genes (Figure 2d). Taking into account the open

reading frame and sequence features such as start and stop codons, the 6-frame peptides can be used to alter currently annotated genes (Figure 2e) and curate new previously unsuspected genes (Figure 2f). This pipeline has significant potential to identify some key biological factors in organisms where they may be missed by traditional means.

1.3 An introduction to phytopathogenic oomycetes

The oomycetes form a heterogeneous eukaryotic group of organisms. Until late in the 20th century, oomycetes were misclassified as fungi due to their similar filamentous morphology, lifestyles and symptoms on plant hosts (Latijnhouwers et al., 2003). It was later confirmed that oomycetes evolved independently of fungi and their similarities were a product of convergent evolution (Money et al., 2004; Hardham, 2007).

Today the agricultural industries and natural environments face threats from a suite of phytopathogenic oomycetes. Destructive phytopathogenic oomycetes include those from the genus *Pythium, Phytophthora, Peronospora, Bremia, Albugo, Aphanomyces, Sclerospora, Lagena* and *Plasmopara* (Le Berre et al., 2008; Blackwell, 2011; Thines and Choi, 2016). The affected plants span from crops, horticulture and native vegetation globally. They contribute to significant losses from reduced crop yield, associated resource wastage (including land, water and fertiliser), pesticide application and destruction of natural ecosystems (Latijnhouwers et al., 2003). The study of these pathogens is becoming more important at the forefront of global research due to their economic and ecological importance.

1.4 *Phytophthora* cell biology

Oomycetes constitute some of the most devastating plant pathogens in the world. In 1845, the world was struck with one of the most impactful famines in history. Losses of up to 75% of potato crops lead to the death of over 1 million people (Fry, 2008). This historic event famously known as the Great Famine was caused by the oomycete pathogen *Phytophthora infestans*. It was at this point that the emergence of increasing crop variety and selective breeding became an essential aspect of worldwide

agriculture. In the 1990's sudden oak death swept across North America and Europe, resulting in the significant loss of old oak and larch forests (Grünwald et al., 2012). The culprit here was *Phytophthora ramorum.* Despite the revolution of genomic and proteomic technologies, these oomycetes amongst many other species are still significant treats today. For example, breeding resistant crop cultivars and oomyceticides applied to combat *P. infestans* disease has improved the outcome of disease, however these pressure on the pathogen have driven emergence of resistant isolates (Ivanov et al., 2021). *P. ramorum* remains an ecological problem in North America and Europe, and the only ongoing treatment to reduce disease load is the application of the chemical phosphite (Grünwald et al., 2012). However similarly to *P. infestans* this has driven rapid evolution of phosphite resistance globally (Hunter et al., 2018a). Therefore such oomycete pathogens are an ongoing problem and require new innovative strategies to manage disease.

There are several attributes of oomycetes that enable their success as phytopathogens. Their dynamic life cycles, pathogenicity and host specificities are the broad aspects that determine their ability to spread, infect and cause disease. The typical polycyclic lifecycle of oomycetes comprises both sexual and asexual components including vegetative mycelia, transient short-lived spores such as zoospores, and intermittent spore-producing/ dormant structures such as sporangia and chlamydospores (Figure 3).

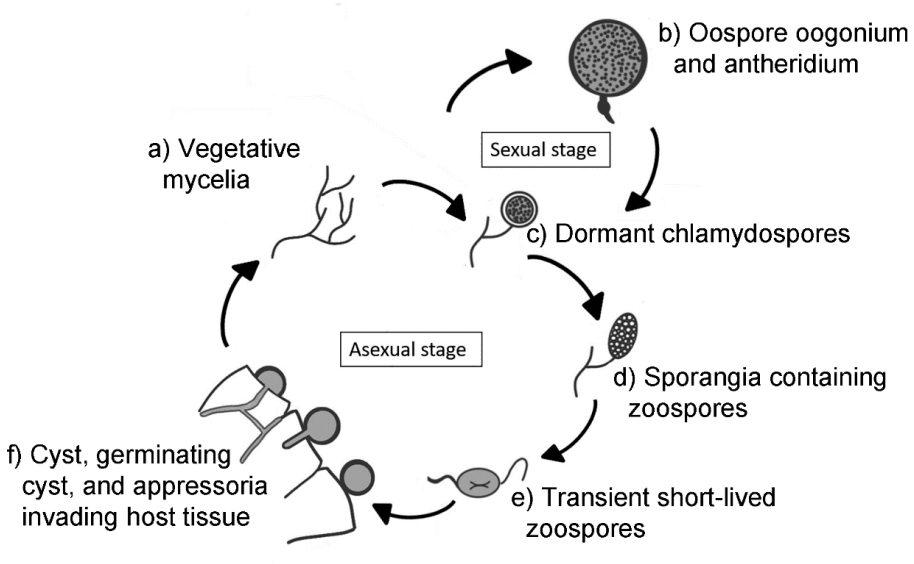

Figure 3. A typical life cycle of phytopathogenic oomycetes.

The life cycle of oomycete species have common characteristics across most species, differing mainly in their target host tissue and specificity. In most *Phytophthora spp*., during less favourable environmental conditions, dormant chlamydospores remain in the soil (Figure 3c) (Hardham, 2005). The dormant cells enable the pathogen to live through harsh environmental conditions and as such, it can easily infect the susceptible host plant once conditions are more favourable (i.e. during warm wet weather occurrences). However, these oomycete pathogens do not require a host to be weakened to achieve infection. The mycelia and chlamydospores subsequently form sporangia, which release free-swimming zoospores (Figure 3d & e). *Phytophthora spp* produces motile zoospores that function as an important component of infection and disease spread (Hardham, 2001; Walker and van West, 2007). This is due to their motility which allows the pathogen to move through aquatic environments and respond to chemical and ionic gradients (Bassani et al., 2020). They can subsequently attach to host tissue and initiate infection. Zoospore motility is controlled by two flagella, the anterior whiplash and the posterior tinsel, and moves towards plant roots by responding to ionic, chemical and nutrient gradients (Van West et al., 2003).

When these cells come into contact with susceptible host roots, they encyst and anchor to the surface of roots via the release of adhesive molecules (Figure 3f) (Gubler and Hardham, 1988). These cysts may form a germ tube, depending on the defence of the plant host, where they can initiate and potentially infect plant host tissue via appressoria-mediated penetration. The mycelia are also able to spread within the plant tissue such as roots, leaves and tubers (depending on species) and transmit between hosts by direct contact (Akino et al., 2014). The outcome of this infection is dependent on the biochemical interaction between the pathogen and the host. Some of these stages have been targets for phytopathogenicity and proteomics studies, representing significant biological interest.

The widely used 'zig-zag' model has shaped how researchers view pathosystems and from which specific models have been constructed such as those for oomycetes (Jones and Dangl, 2006; Hein et al., 2009). Pathogen-associated molecular patterns are detected by host pattern recognition receptors in the host and can trigger pathogen-triggered immunity (PTI). To overcome PTI, the pathogen may then deliver effector molecules to interfere with host immunity which in turn may drive effector-triggered susceptibility (ETS) or be recognised and cause effector-triggered immunity (ETI) by corresponding resistance genes (i.e. R-Avr interactions). Break down in ETI occurs via mutations in the Avr effector leading to non-recognition (Hein et al., 2009). Single R-Avr gene interactions that result in plant resistance (qualitative resistance) have been observed in several oomycete pathogens with specific hosts. For example, the phytopathogenic oomycete *Plasmopara viticola,* the causal agent of downy mildew, has demonstrated qualitative resistance to its grapevine host. Emergence of *P. viticola* isolates are able to overcome the single dominant resistance gene *Rpv3* in specific varieties of grapevine and cause disease (Peressotti et al., 2010). Quantitative resistance is considered more durable and frequent due to its emerged and ongoing evolutionary implications (Lindhout, 2002). However, in other isolates of *P. viticola* in relation to a range of grapevine cultivars, quantitative resistance is the predominant type of interaction observed due to the continual implementation of resistance genes (Heyman et al., 2021). Quantitative resistance may result in evolutionary gain or loss of effectors by pathogens. More recent models such as the 'invasion model' cover a wider range of symbioses, referring to the detection of invasion patterns and how co-evolution between invasion patterns and their recognition directs plant-pathogen interactions (Cook et al., 2015).

Effectors and resistance gene products have been widely capitalised for the development of resistance crops through the introduction of R genes into crops. Defining pathogenic factors is commonly achieved through gene prediction and forward/reverse genetics, which aim to identify virulence factors expressed by a plant pathogen (Jones et al., 2018). Identification of gene families with roles in infection and virulence can be exploited for applications such as resistance gene inclusion and targeted management (Morgan and Kamoun, 2007). A reduction in the cost of whole

genome sequencing results in an increasing number of phytopathogenic oomycete genomes being sequenced. The transcriptome and proteome are not fully correlated, as transcripts do not inform on localisation of their protein products unless they are obtained from some specific tissue types (Tyers and Mann, 2003). Additionally, although gene expression can be quantified, final protein abundance cannot be predicted from transcripts. Assessing protein content complements and validates transcript data, creating a more accurate representation of the biochemistry and biology of an organism (El-Aneed et al., 2009).

1.5 Proteomic methods to elucidate oomycete sub-proteomes

The polycyclic life cycle of *Phytophthora* species comprises specialised cell types that each function in different ways towards the goal of successful infection and spread. Comparative proteomics is useful in characterising the biochemical differences in these sub-proteomes and highlighting key biological features. *Phytophthora palmivora* infects a broad range of tropical fruit and nut plants. Its contribution to cacao crop losses amounts to global losses of nearly $4 billion USD per annum (Bailey and Meinhardt, 2016).

The cell-specific proteomes of *P. palmivora* in the mycelia, sporangia, zoospores, cysts and germinating cysts were examined to identify differentially abundant proteins using 2D-PAGE followed by MALDI-TOF analysis of tryptic peptides and spectral matching to predicted genes. This study revealed that approximately one per cent of a total of 400 protein gel spots were differentially abundant in all developmental stages (Shepherd et al., 2003). Upon identification, proteins differentially expressed in the zoospores included actin isoforms indicating cytoskeletal development during zoosporogenesis. The differentially abundant proteins found in the cysts were predominantly actin plaques. These are associated with plug deposition in germ tubes where the appressoria eventually form and penetrate the host tissue (Shepherd et al., 2003; Kots et al., 2017). Actin plaques have been described in non-motile stages of the *Phytophthora* lifecycle such as the hyphae, young cysts and surrounding sporangia playing roles in morphogenesis (Ketelaar et al., 2012; Kots et al., 2017).

As advances in LC-MS/MS technology have surpassed the sensitivity and accuracy of MALDI instrumentation, the depth of microbial proteome analysis has exponentially increased. Proteins in the cysts and oospores have been investigated in *P. sojae* using TMT labelling followed by LC-MS/MS. *P. sojae* causes stem rot in soybean and various lupin varieties. The cysts were abundant in proteins related to lipid metabolism and fatty acid degradation (Zhang et al., 2020a). This correlates to similar findings in *Phytophthora capsici,* a broad-range pathogen that causes blight and fruit rot in many crops including capsicum, eggplant, and tomato (Pang et al., 2017). The mycelia and cysts of *P. capsici* were labelled with iTRAQ reagents, separated by strong cation exchange HPLC and analysed by LC-MS/MS. This suggests that the early stages of infection are reliant on using fatty acids for energy production to form subsequent structures such as the appressoria (Pang et al., 2017). Nitrogen metabolism, increased translation and biogenesis were also found in *P. sojae* which supports the initiation of infective structures by responding to environmental signals and forming these structures (Grenville-Briggs et al., 2005; Zhang et al., 2020a).

Similarly, the oospores of *P. capsici* showed abundance in RNA transport, protein processing, translation initiation, protein synthesis and folding indicating a high rate of translation to support germination (Zhang et al., 2020a). These are just some examples of how proteomics has been used to investigate metabolic differentiation between cell types.

1.6 Unravelling effectors through the use of proteomics

Secreted molecules such as effectors target different aspects of the plant system to overcome the host defences. Effector discovery in oomycetes is primarily achieved by bioinformatically screening gene models for conserved motifs, particularly the RxLR and Crinkler (CRN) motifs (Ellis et al., 2009). RxLR genes play roles in virulence in some oomycetes (*Phytophthoras* and downy mildew species) by facilitating the translocation of effectors into host cells. RxLRs are not involved in virulence in all phytopathogenic oomycetes and are not found in necrotrophic oomycetes such as the *Pythium spp* (Anderson et al., 2015).

Similarly, CRN motifs also mediate the transport of effectors into host cells (Schornack et al., 2010). Members of the RxLR and CRN families can translocate into host cells, promote nutrient leakage in the host and allow for pathogen dispersal and successful colonisation (Jones and Dangl, 2006). Subsequently to the discovery of these signatures, fungal models were used to search for effectors and effector-like molecules, including screening for cysteine-rich small proteins and signal peptides (Sperschneider et al., 2015; Selin et al., 2016). Some of these effectors are recognised by corresponding R genes expressed by the host (Kamoun, 2006). For example, a potato R gene (*R1*) has been cloned and successfully implemented into potato crops to block infection by recognising the corresponding *P. infestans* effector molecule *Avr1* thus preventing the pathogen from successfully infecting the host (Ballvora et al., 2002). Predicted and candidate effectors are typically tested by implementing techniques such as effector gene deletion, transformation and transient expression into a susceptible hosts (Bozkurt et al., 2012; Sharpee and Dean, 2016; Liu et al., 2018).

A secreted CRN necrosis-inducing protein (CRN2) was also identified in *P. infestans* in the cysts and at the site of appressoria formation using 2D SDS-PAGE separation followed by LC-MS/MS (Ebstrup et al., 2005). CRN2 is a known cell death inducer, in hosts such as tobacco and tomato (Torto et al., 2003; Du et al., 2015). A high abundance of CRN2 in the cysts and the site of penetration suggests it interacts with components at the host interface which determines the outcome of recognition and infection (Torto et al., 2003). It is a key component of important cell stages like the cysts with their necrotic-inducing proteins that are crucial in profiling the virulence of such nasty pathogens.

Secretome enrichment has been a useful method for elucidating the extracellular protein content of a pathogen. This is typically achieved by concentrating the culture filtrate of oomycetes grown *in vitro* with or without exposure to plant material to trigger the release of secreted molecules (Meijer et al., 2014; Severino et al., 2014). Secretome analysis by LC-MS/MS of *P. infestans* led to the identification of 20 members of the RxLR family, 13 members of the CRN family and 5 necrosis-inducing proteins, many of which are required for virulence (Meijer et al., 2014). Additionally,

several elicitin proteins including INF1, INF2A-like, INF4, INF5A and INF7 were identified in high abundance. Elicitins such as these are highly conserved small molecular weight extracellular toxins secreted by oomycetes that induce an immune response in hosts (Qutob et al., 2003; Du et al., 2017). Elicitins are well known to trigger a hypersensitive response in plants as a pathogen-associated molecular pattern (PAMP) (Horta et al., 2008). In some plant species such as tobacco, elicitins such as INF1 are avirulent as they induce a gene for gene hypersensitive response through the activation of jasmonic acid production, which causes localised cell death to stop the spread of disease (Kamoun et al., 1998).

P. plurivora has a wide host range and primarily infects native beech trees throughout European forests (Schoebel et al., 2014). To identify virulence factors, *P. plurivora* was exposed to beech root exudate and the culture filtrate was analysed using shotgun proteomics (Severino et al., 2014). In conjunction with various bioinformatics tools such as SignalP and BLAST, protein functions within the secretome were determined. A follow-up quantitative analysis examined changes in proteins of *P. plurivora* infecting plant roots. Along with the identification of known Avr effectors such as NLP and Avr1b-1, proteins belonging to the necrosis and ethylene-inducing protein I family (NEP1), and several proteins with the RxLR effector motif were identified. NEP1 proteins have been characterised in *P. infestans* and *P. megakarya,* and play important roles in inducing necrosis in their hosts, tobacco and cacao, respectively (Bae et al., 2005; Kanneganti et al., 2006).

The extracellular proteomes of *Phytophthora chlamydospora P. gonapodyides and P. pseudosyringae* were examined by LC-MS/MS. Several virulence-associated molecules were identified (McGowan et al., 2020). These included elicitor proteins, cysteine-rich secreted proteins, necrosis-inducing proteins, hydrolase, carbohydrate esterases and binding molecules. In *P. capsici*, LC-MS analysis confirmed the roles of extracellular vesicles in the transport of effectors thus facilitating the delivery of these molecules into the extracellular space (Fang et al., 2021). The outcome of oomycete-host plant interactions is predominantly dictated by Avr effector-host R gene interactions. Therefore, undefeated R genes can be implemented into the host system to achieve

disease resistance. Effectors with no known corresponding R gene pose a significant challenge in breeding resistant cultivars of host plants. For example, the *P. sojae* intracellular effector PsAvh238 suppresses biosynthesis of ethylene which is an important constituent of plant defence against hemibiotrophs and necrotrophs (Fabro, 2022). There is no known R gene for this effector in any of *P. sojae's* hosts, therefore poses a significant problem in the soybean industry (Yang et al., 2019).

1.7 Energetics and signal transduction for cellular differentiation, growth and motility

Mycelia must optimise energy generation and metabolism to drive the efficient switch between cell states and support the formation of rapidly growing structures (Bartnicki-Garcia et al., 1983) (Figure 3a). To support the saprophytic survival of oomycetes within host tissue, there must be a continual influx of nutrients to drive energy metabolism for hyphal growth (Andronis et al., 2022). In zoospores, energy is used to drive a completely different process. Zoospore motility is an energy-intensive process in addition to subsequently forming cysts and appressoria to germinate within host tissue (Bhadauria et al., 2009). Components of zoospores such as the dynein complex which are the main drivers of the motor complex are energy-dependent (Wickstead and Gull, 2007; Narayan et al., 2010). Signal transduction in zoospores requires efficient and quick use of energy for changes in direction in response to quick-changing environmental chemotactic gradients as shown in *P. sojae* (Zhang et al., 2020b).

Zoospores differentiate into cysts where they release adhesins to facilitate attachment to the host cell surfaces (Hardham, 2001; Shan et al., 2004). The germinating cyst and mycelial proteomes of *Phytophthora pisi* and *P. sojae* were examined using excised bands from a 1D-gel proteome analysis followed by MS (Hosseini et al., 2015). The mycelial and cyst proteomes were separated on a 1D SDS-PAGE where gel lanes containing the fractionated proteome were excised, and proteins were subsequently extracted and compared using a label-free approach. A total of 2128 cyst and 2275 hyphal proteins from *P. sojae* and 2197 cyst and 2335 hyphal proteins from *P. pisi* were identified. In both species, proteins such as enoyl-CoA hydratase, carnitine acetyltransferase and acyl-CoA dehydrogenase were more abundant in germinating cysts. These are primarily involved in lipid transport, metabolism and energy

production through the beta-oxidation pathway (Shen and Burger, 2009). Upon successful infection, it is expected that the pathogen will have unrestricted access to carbon and other forms of nutrients obtained via the host. Similarly, the hyphae had enriched secondary metabolite biosynthesis and catabolism, carbohydrate transport and catabolism, which corresponds with obtaining nutrients from the plant as its primary energy source, which in turn are involved in the production of infective structures when required to further facilitate host penetration (Savidor et al., 2008; Hosseini et al., 2015). The differentiation of metabolic, energy and focus of cellular processes are highly specialised in the different cell types of phytopathogenic oomycetes and are informative on the mechanisms of successful infection.

Phosphorylation is a key post-translational modification that can be used in enzyme and signalling activation (Rubin and Rosen, 1975). To determine the phosphorylation state of proteins throughout the different life stages, sub-proteomes of the mycelia, sporangia, zoospores, cysts, germinated cysts and appressoria of *P. infestans* were analysed using phosphoproteomics (Resjö et al., 2014). Sub-proteomes were separated using 1D SDS-PAGE, entire lanes excised and proteins digested. Phosphoproteins were subsequently quantified using a label-free MS approach. This set-up identified differentially abundant phosphoproteins that play important roles in signalling to initiate cellular differentiation, formation of infectious structures and virulence. The unique phosphopeptides found in the appressoria, the structure that penetrates host tissue, are composed mostly of transporters such as folate-biopterin, ATP-binding cassettes, glucose transporters and an abundance of amino acid and auxin permeases were found in the appressoria as per the assigned Uniprot Gene Ontologies. This is likely due to the rapid formation of cysts from appressoria, where upregulation of amino acid biosynthesis and auxin permeases are required for this cellular differentiation.

These differential phosphorylation states suggest increased translocation of molecules which could be needed to penetrate the host cells and overcome host immune responses quickly to establish infection (Resjö et al., 2014). Additionally, several members of the CRN effector families were phosphorylated in all the other stages of

the *P. infestans* life cycle including the mycelia. This suggests that CRN proteins may have roles other than host manipulation such as they are maintained and phosphoylated in these pre-infective cells.

1.8 Combatting host plant defence

Pathogenic oomycetes require an organised response to host defence. These responses aim to dampen the host defence response and detoxify the destructive molecules that are produced by the plant. Aside from effector-mediated infection, the production of defence-related proteins assists the pathogen in overcoming host defence. In oomycetes, infective structures such as appressoria and haustoria (Figure 3f) are the key to understanding the initial defence responses as they represent the initial penetration efforts of the pathogen into the host tissue (Boevink et al., 2020).

A study using 2D-PAGE was performed to identify differentially abundant proteins between *in vitro* cysts, germinated cysts and appressoria, along with *in planta* development of appressoria in *P. infestans* (Ebstrup et al., 2005). The proteins upregulated in the cysts included those associated with energy metabolism, for example, homologous to acyl-CoA synthetases, which is a key factor in the catabolism of fatty acids. Thioredoxin peroxidase was also found to be upregulated, having roles in the detoxification of reactive oxygen species (ROS). This is likely abundant in the cysts as the production of ROS is an important aspect of plant defence and therefore important that the pathogen can avoid and reduce exposure to ROS during the early infective stages of infection (Thordal-Christensen et al., 1997). Proteins increased in abundance in the appressoria, including a long chain acyl-CoA dehydrogenase, were directly involved in the energy metabolism required for the formation of these penetrative structures and the generation of turgor pressure. Similarly, formate dehydrogenase was highly abundant, which forms part of the detoxification system to remove formaldehyde from methanol and ethanol catabolism (Vorholt, 2002).

1.9 Understanding targets for chemical control using proteomics

Similarly to fungicides, oomyceticides have varying targets on the pathogen that inhibit their growth. These can include cell walls, membranes and intracellular

components, essential genes for survival and that if targeted, disrupt normal functioning and can be used to control disease (Theis and Stahl, 2004). Oomyceticides typically act on the mycelial life stage and affect mycelial growth and zoospore production and are applied to host plants at varying times depending on the causal agent (Cohen and Coffey, 1986). Resistance oomycetes to chemical control agents typically emerge through long term application, particularly for single target oomyceticides (Gisi et al., 2015).

The mode of action of oomyceticides can be determined by transcriptomics and proteomics (Cools and Hammond-Kosack, 2013; Pang et al., 2015). Transcripts and proteomes can inform on comparative changes of the transcriptome and proteome respectively between untreated and treated organisms along with comparing isolates and varieties that have differing sensitivities to the chemical (Shang et al., 2021). Proteomics has been used to understand the depth in which these chemicals interact with the pathogen along with non-target and downstream effects.

Pyrimorph is a morpholine compound that inhibits mycelial growth, production of sporangia, cyst germination and cell wall biosynthesis in several species of *Phytophthora* (Yan et al., 2010). It impairs aspects of energy generation which influences all stages of life, similar to other oomyceticides such as Cyazofamid and Fluzinam used for other *Phytophthora spp.* Pyrimorph is used to combat blight and rot of many commercial peppers and other important crops caused by *P. capsici.* To identify the biochemical pathways that are disrupted, the proteome of *P. capsici* was analysed when treated with a sub-lethal dose of the chemical agent (Pang et al., 2013, 2015). Pyrimorph-sensitive and -resistant *P. capsici* isolates were treated and their respective proteomes were analysed by iTRAQ labelling followed by LC-MS/MS detection/quantification. The significant differentially abundant proteins between the treated and untreated pyrimorph-sensitive isolate were related to cell wall biosynthesis from their assigned KEGG pathways (Pang et al., 2015). Using proteomic data, this study suggested that pyrimorph inhibits cell wall synthesis and was subsequently confirmed using a crystalline cellulose synthesis assay where cellulose synthesis was substantially inhibited by the oomyceticide. Additionally, changes in the

structure of the cell wall were confirmed by microscopy, showing distortions and swelling of mycelial structures (Yan et al., 2010). Proteins with gene ontologies associated with energy production and respiration such as ATP synthase and enoyl-CoA hydratase were also differentially abundant showing that pyrimorph interferes with energy metabolism. Respiration of *P. capsica* was determined by measuring oxygen consumption and showed significant inhibition (Yan et al., 2010). This suggests that energy metabolism could be a downstream effect of pyrimorph treatment.

The fungicide SYP-14288 (2,4-dinitro-5-chloro-[1-(2,6-dichloro-4-nitroaniline)]-toluene) is another broad-spectrum chemical agent used to control fungal and oomycete plant diseases such as those caused by *P. capsici* and *P. infestans* (Wang et al., 2018, 2021). Quantitative proteomics suggested this novel chemical interrupts several core processes of the pathogen according to their assigned orthologue groups (Cai et al., 2019). The pathways significantly affected by exposure to SYP-14288 were mitochondrial respiration, amino acid metabolism, fatty acid metabolism and the citric acid cycle. The changes in energy metabolism in *P. capsici* in response to SYP-14288 cause downstream homeostatic disruption of ATP biosynthesis and disruption of ATP regulation (Wang et al., 2018; Cai et al., 2019). Relative expression of core genes representative of the gene ontologies comprising these significantly differentially abundant proteins showed comparable trends. This included several genes involved in metabolism, mitochondrial respiration and energy generation (Cai et al., 2019).

Phytophthora cactorum causes root rot in over 200 plant species, including some economically important crops such as apple orchards. It is commonly treated with zoxamide, which binds to β-tubulin and inhibits nuclear division in germinating cysts (Young and Slawecki, 2001). Similarly to the effects of pyrimorph on *P. capsici,* comparative proteomic analysis using 2D LC-MS/MS revealed that *P. capsici* treated with zoxamide showed a significant reduction in the abundance of cytoskeleton-related proteins (Mei et al., 2014). However, in response to zoxamide, detoxifying enzymes such as glutathione reductase and catechol O-methyltransferase accumulated in the proteome. A subsequent study suggested similar detoxifying enzymes such as glutathione transferase were found to be responsible for reduced

sensitivity of *P. cactorum* to the fungicide as an artefact of long-term exposure (Mei et al., 2015).

These examples demonstrate the potential for exploitation of cell components such as cell wall proteins for the use of chemical management is understudied. Widespread resistance has been observed with a large number of traditional oomyceticides along with observed rapid evolution conveying tolerance in many *Phytophthora* spp (Ziogas et al., 2006; Hunter et al., 2018b). There is an urgent need for the constant development of new management strategies and perhaps with more proteomic data of subcellular components such as cell walls, new targets will be identified. In addition, there is a lack of proteomic data surrounding the chlamydospore and oospore structures of oomycete cell cycles. These are dormant structures that have specialised cell walls to avoid degradation during dormancy with varying metabolic activity (Hemmes and Wong, 1975). Chlamydospores and oospores could contain biochemical targets however, there is a lack of representation of these cells as they are difficult to produce *in vitro*. Taken together, the identification and quantification of sub-proteomes and cell-specific proteomes provide a more in-depth understanding of oomycete infection and can aid in the development of sustainable control solutions.

1.10 *Phytophthora cinnamomi,* the causal agent of native dieback

Of the *Phytophthora* species, *P. cinnamomi* has been named one of the most significant phytopathogenic oomycetes globally (Kamoun et al., 2015). It is believed to have originated from Papua New Guinea and was first isolated in 1922 from the cinnamon tree (Hardham, 2005). Since, it has been found globally, causing significant environmental and economic impacts. *P. cinnamomi* is a root rotting pathogen that affects a wide range of native trees and horticultural crops such as avocado, pineapple, macadamia and stone fruits (Hardham and Blackman, 2018).

P. cinnamomi adheres to the typical oomycete polycyclic life cycle (Figure 3). The mycelia grow vegetatively within plant tissue and produce dormant spores or spore-containing structures (sporangia). These subsequently release zoospores that adhere to plant root tissue upon contact. Unlike other *Phytophthora species* such as *P.*

infestans, P. cinnamomi can only spread via root-to-root contact between infected and uninfected plants and by encysted zoospores on plant roots. It cannot be transmitted via other parts of the plant such as leaves. Although this pathogen begins infection at the roots of plants and resides within the root system, symptoms appear at the canopy exhibiting wilting leaves and loss of foliage (Figure 4). As disease severity progresses, the symptoms work their way down to the lower canopy of the plant (Weste and Marks, 1987).

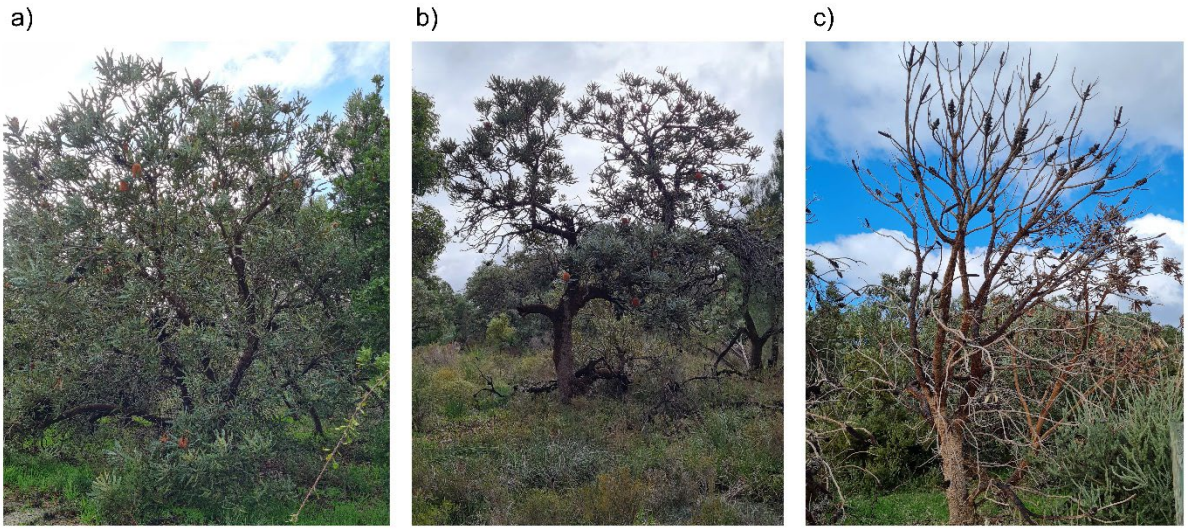

Figure 4. Progression of dieback disease. **a)** A healthy native Australian tree (Banksia). **b)** A Banksia tree exhibiting symptoms of dieback disease in a confirmed area of *P. cinnamomi* infestation. **c)** A dead Banksia tree with a known cause of death of dieback disease.

In Australia, approximately 40% of native vegetation species are susceptible to infection by this pathogen (Shearer et al., 2004). As Western Australia has some of the most diverse ranges of vegetation globally, this equates to a high proportion of infected and at-risk ecosystems and thus dieback disease has caused the loss and destruction of vast areas of natural vegetation. Key susceptible native plants include species from the *Banksia, Pinus, Eucalyptus* and *Xanthorrhoea* genus' (Bullock et al., 2000; Hardham and Blackman, 2018). Additionally, avocados, of which Australia is a large producer, are one of the many horticultural crops under threat by *P. cinnamomi*.

The genomic resources available for *P. cinnamomi* include a publicly available draft genome of the MU94-48 isolate, a model Australian isolate (Aghighi et al., 2016). Five other isolates of *P. cinnamomi* from Australia, New Zealand and South Africa have published genomes (Studholme et al., 2016; Longmuir et al., 2018; Engelbrecht et al., 2021) (Table 2).

The MU94-48 draft genome is publicly available (available at https://mycocosm.jgi.doe.gov/Phyci1). Compared to other *P. cinnamomi* isolates and other *Phytophthora* species, this draft genome has a higher number of predicted genes.

Table 2: Genome comparison of currently sequenced *P. cinnamomi* genomes.

Isolate	Location	Genome size (Mbp)	Coverage	No. scaffold	N50 (Mb)	No. gene models	Sequencing and assembly	Reference
MU94-48	Australia	77.97	69.6x	1314	0.26	26131	Illumina, 454, Sanger	JGI mycocosm
DU054		62.80	300x	14268	.001	23414	Illumina, IDBA-UD	Longmuir et al., 2018
WA94.26		68.07	300x	10084	0.02	22573	Illumina, IDBA-UD	Longmuir et al., 2018
MP94-48	New Zealand	53.69	35x	5777	0.02	-*	Illumina, SPAdes, SSPACE	Studholme et al., 2016
NZFS 3750		53.97	30x	6270	.02	-*	Illumina, SPAdes, SSPACE	Studholme et al., 2016
GKB4	South Africa	109.7	100x	133	1.8	19981	Oxford Nanopore, Illumina	Engelbrecht et a., 2021

*annotation not available.

The molecular mechanism of *P. cinnamomi* pathogenicity has been investigated to a limited extent. Two elicitins have been characterised to play a role in virulence. Elicitins are highly conserved small molecular weight extracellular toxins secreted by oomycetes that induce an immune response in hosts (Qutob et al., 2003; Du et al., 2017). Elicitins were first observed in 1985 and characterised in 1988 by chromatographic purification, amino acid analysis and necrosis assays (Bonnet and Rousse, 1985; Billard et al., 1988). The two *P. cinnamomi* elicitins α- and β-cinnamomin assist in the infection process and can evade recognition by most plant hosts (Hardham, 2005; Horta et al., 2010; Oßwald et al., 2014; Islam et al., 2019).

There are several difficulties surrounding the management and control of dieback disease. While in some systems the transformation of R genes is implemented, this is less applicable in *P. cinnamomi*. Although it does infect a variety of crops, a major concern for dieback disease is in natural vegetation (Shearer et al., 2004; Cahill et al., 2008). If avirulence genes were found in *P. cinnamomi,* they could potentially be exploited through the discovery and mobilisation of novel R genes in crops such as avocados. However, in the masses of global native vegetation that are affected, this is not feasible.

In this case, other methods of control must be implemented such as the application of phosphite. Inorganic phosphites are reduced forms of phosphate and are commonly used as pesticides to manage dieback disease (Smillie, 1989; Eshraghi et al., 2011). Phosphite is applied to susceptible plants by foliar spray or by injecting directly into the trunk where it subsequently circulates throughout the plant system (Masikane et al., 2020). Despite its widespread use, the mode of action of phosphite is not well understood. It has been suggested that it acts directly on the pathogen and indirectly through that plant host and phosphite resistance is a growing issue (Smillie, 1989; Lim et al., 2013a). Firstly, it was previously shown that phosphite can directly inhibit mycelial growth of *P. cinnamomi in vitro*. However, the sensitivity of *P. cinnamomi* to phosphite varies between isolates. For instance, 70 isolates were screened for phosphite sensitivity *in vitro,* of which 9% were sensitive, 82% had intermediate sensitivity and 9% were tolerant (Wilkinson et al., 2001). Phosphite tolerance has

increasingly been observed in areas with prolonged use such as horticultural plantations (Dobrowolski et al., 2008; Hunter et al., 2018b, 2022).

Several studies suggest that phosphite also acts indirectly by priming the plant defence response (Smillie et al., 1989; Eshraghi et al., 2011; Lim et al., 2013b). In *P. infestans* potato hosts, proteins such as glutathione S-transferase, peroxidase, protease inhibitors and components of the salicylic acid pathway are involved in microbial defence (Lim et al., 2013a). This has been observed in similar studies where transcription of defence genes and protein abundance of salicylic acid and jasmonic acid were increased in abundance in potato and *Arabidopsis thaliana* as a result of phosphite treatment (Eshraghi et al., 2011; Machinandiarena et al., 2012; Feldman et al., 2020). Despite these observations, the mode of action in which phosphite inhibits the growth of *Phytophthora* has not been defined, nor have the mechanisms of phosphite primed plant defence.

With the increasing pressure from emerging chemical resistance, there is a need to understand the mechanisms in which phosphite interacts with the pathogen and plant and identify the biochemical source of resistance. This highlights the need for new innovative management plans that can minimise the spread and disease in such systems.

1.11 Thesis hypotheses and aims

There are two overall goals of this work. The first was to gain an understanding of the biochemical mechanisms of pathogenicity in *P. cinnamomi*, and the second was to elucidate the mode of action of the chemical control agent phosphite.

As a precursor to elucidating the biochemistry of this pathogen and the effects of phosphite, the genome needs to be assessed The MU94-48 draft genome has a higher number of predicted genes than expected compared to other isolates and species and as such, could potentially contain annotation errors and may not be fully representative of the detectable proteome. Shotgun proteomics relies on accurately

annotated genes for spectral matching, therefore the MU94-48 annotation must be investigated to determine how it compares to the detected proteome.

Proteomics allows us to take a biochemical snapshot of an organism in any particular state and measure altered protein abundance in different states. It has been used to understand the virulence of many important oomycete plant pathogens and reveal key factors that can subsequently be exploited for new and innovative disease management strategies. This type of workflow has not yet been applied to *P. cinnamomi*. It has been described mostly in regards to its life cycle, morphology and its general impact in an ecological and horticultural context. The surface has barely been scratched in terms of the intricacies of its pathogenicity. Following the trends observed in other *Phytophthora* species, a plethora of virulence-associated molecules is expected to be woven throughout the biochemistry of this destructive pathogen. With the lack of biochemical representation of *P. cinnamomi* in the literature, a shotgun proteomic approach can be used to investigate its nuances.

Phosphite is the only control option to combat dieback disease and its mode of action remains elusive. There is also increasing pressure from the emergence of phosphite-resistant isolates (Dobrowolski et al., 2008; Hunter et al., 2018b, 2022). The main isolate of interest used in this work was MU94-48, which is prolific in both natural ecosystems and horticultural plantations in Western Australia (Aghighi et al., 2016). In light of the gaps in the literature and the available resources, three hypotheses were formulated:

Hypothesis 1: The draft genome of *P. cinnamomi* MU94-48 is incomplete and the current gene annotations are not fully representative of the proteome.

Aim – To use mass spectra obtained from representative sub-proteomes derived from different stages of the *P.* cinnamomi MU94-98 life cycle to improve the draft genome annotation of isolate MU94-48 (Chapter 3).

Hypothesis 2: *P. cinnamomi* sub-proteomes produce specialised virulence factors that contribute to its pathogenic success.

Aim: To investigate the differential proteomes of the mycelia, zoospores and secretome and identify important biochemical aspects of virulence (Chapter 4).

Hypothesis 3: Phosphite exhibits bimodal action towards the pathogen and the host.

Aim: To confirm and understand the direct and indirect mechanisms of phosphite and identify the differences inflicted between sensitive and tolerant isolates (Chapter 5).

Chapter 2: General Materials and methods

2.1 Growth and maintenance of *Phytophthora* isolates

2.1.1 Isolate stocks

P. cinnamomi isolates MU94-48 and CPSM366 were obtained from the Centre for Phytophthora Science and Management, Murdoch University, Western Australia. MU94-48 was collected from susceptible *Eucalyptus marginata* in Willowdale, Western Australia with no history of phosphite use and CPSM366 was collected from a Western Australian avocado orchard with a history of extensive phosphite use and reduced efficacy of protection on dieback was observed (Dobrowolski et al., 2008; Simamora et al., 2017). Stock plugs were rejuvenated by passage through *Malus domestica* cv. Granny Smith fruit, which are used in as a readily available model in sterile laboratory conditions to promote pathogenicity in *P. cinnamomi* when stored long-term *in vitro* (Crone et al., 2013). Stock plugs were stored in sterile water in McCartney bottles at room temperature prior to use.

2.1.2 Growth and isolation of MU94-48 and CPSM366 mycelia

For mycelial growth, stock plugs were placed on V8 agar plates and incubated at room temperature in the dark for four to seven days. For the growth of mycelia in liquid media, four days of mycelial growth were scraped from a V8 plate and inoculated into 50 mL of Riberio's minimal media supplemented with 25 mM glucose (Ribeiro, 1979). Flasks were incubated for 3 days at 24°C in the dark. Mycelia were isolated by centrifugation. The mycelial pellet was washed twice with MilliQ water and observed microscopically to confirm that hyphal cells predominated. Purified mycelia were snap frozen in liquid nitrogen, freeze-dried and stored at -80°C until use.

2.1.3 Growth and isolation of MU94-48 zoospores

Zoospores were produced as previously described (Byrt and Grant, 1979). Briefly, mycelia were grown on V8 agar plates with a 5 cm x 5 cm sheet of miracloth on the surface and incubated for 4 days at 24°C in the dark. The mycelial mat was transferred into V8 liquid media and incubated on a shaker at 100 rpm for 24 hours at 24°C under

fluorescent light. The mycelial mat was subsequently washed three times in a sterile solution of 10 mM calcium nitrate, 5 mM potassium nitrate, 5 mM magnesium sulphate and 1:1000 v:v chelated iron solution and further incubated in this solution on a shaker at 100 rpm for 24 hours at 24°C under fluorescent light. The mycelial mat was transferred to a petri dish and incubated for 1.5 hours in water at room temperature followed by 30 minutes at 4°C for the formation of sporangia and release of zoospores. The release of zoospores was observed under the microscope and the zoospore suspension was carefully passed through a glass wool syringe to remove any mycelial fragments. Zoospores were harvested by centrifugation at 3000 g for 30 minutes, observed microscopically to ensure purity, and counted with a haemocytometer. This incubation process was repeated until sufficient zoospores were harvested. $4.8E^5$ spores were used for each biological replicate. Purified zoospores were snap frozen, freeze-dried in liquid nitrogen and stored at -80°C until use.

2.1.4 Isolation of MU94-48 secretome

The secretome was purified from mycelia grown in liquid minimal media (see 2.1.2 'Growth and isolation of MU94-48 and CPSM366 mycelia'). The culture filtrate was obtained from the mycelia after three days of growth. The supernatant from the centrifuged mycelia was decanted and filtered sterilised with a 0.22 µm syringe filter.

2.2. Glyceraldehyde phosphate dehydrogenase (GAPDH) assay for intracellular contamination

GAPDH can be used as an intracellular enzyme marker. To determine the amount of intracellular contamination in the secretome, 30 µg of each sub-proteome was used to determine GAPDH enzymatic activity as per the manufacturer's instructions (Sigma, St Louis, USA).

2.3. Phosphite and *Lupinus angustifolius* handling

2.3.1. Growth of MU94-49 and CPSM366 on phosphite media

MU94-98 and CPSM366 plugs were inoculated onto modified minimal media supplemented with 0.0 µg/mL, 2.5 µg/mL, 5 µg/m, 7.5 µg/mL, 10.0 µg/ml, 20.0 µg/mL,

40 µg/mL, 100 µg/mL, 500 µg/mL and 1000 µg/mL of phosphite (phosphorous acid) (Sigma, St Louis, USA). Mycelial cultures were incubated at room temperature in the dark (Hunter et al., 2022). After 14 days of growth, the mycelial radial growth was measured to determine growth inhibition. The EC_{50} and minimum inhibitory concentration (MIC) for both isolates were obtained by plotting the percentage of growth inhibition against the concentration of phosphite as previously described (Lóopez-Ruiz et al., 2010). 0.0 µg/mL and 2.5 µg/mL were used for further experimentation as these represented an untreated and a sub-lethal dose of phosphite causing a physiological effect and providing enough biomass for experimentation Proteins were extracted as per section 2.4 'Protein extraction'.

2.3.2. Growth of *P. cinnamomi* isolates for lupin root inoculation

A ring of 5 mm sterile discs of Whatman paper were placed on V8 agar plates approximately halfway between the centre and edge of the plates. Plates were inoculated with plugs of MU94-48 or CPSM366 isolates of *P. cinnamomi.* After 7 days of growth, the discs overgrown with mycelia were used to inoculate lupin roots.

2.3.3. Germination and growth of *L. angustifolius* seedlings

Lupinus angustifolius ('Tanjil' narrow leaf Lupin) presents as a good model for this study as it grows rapidly, highly susceptible to dieback and it has a published genome sequence required for proteomics work (Qiu et al., 2014; Garg et al., 2022). *L. angustifolius* seeds were surface sterilised with 5% sodium hypochlorite, washed twice with 70% ethanol and washed three times with water. Seeds were placed in clear containers lined with moist Whatman paper and left to germinate for 3 days at room temperature under natural light. Subsequent days of growth were kept on a light shelf at room temperature with a photoperiod of 12 hours (Smillie et al., 1989).

2.3.4. Inoculation of *L. angustifolius* with *P. cinnamomi*

Mycelial discs from *P. cinnamomi* isolates MU94-48 and CPSM366 were used to inoculate *L. angustifolius* seedling. Discs of mycelia were placed under the root tips of three-day-old lupin seedlings. Infected roots and uninfected controls were harvested

at one and three days post- inoculation (see section 2.5.3 'Protein purification of root tissue by chloroform-methanol precipitation').

2.3.5. Phosphite treatment of *L. angustifolius*

After three days of growth, lupin seedlings were sprayed with a solution of 0.5% pH 7 solution of phosphite (Sigma, St Louis, USA) based on commercial phosphite solution concentrations (Barrett et al., 2003; Cerioni and Rapisarda, 2013). Treated seedlings were transferred to a clean container and placed back on the light shelf.

2.3.6. Phosphite treatment and inoculation of *L. angustifolius* with *P. cinnamomi*

L. angustifolius seeds were surface sterilised and germinated as per section 2.3.3 'Germination and growth of *L. angustifolius* seedlings'. To test the effects of phosphite on the colonisation ability of *P. cinnamomi* on the plant tissue, phosphite-treated lupin was inoculated with mycelial discs of the two *P. cinnamomi* isolates and after three days, lesion scores were recorded. Using untreated lupin as a model, both isolates of *P. cinnamomi* unanimously result in visible lesions therefore this presents as an ideal model for *in planta* experimentation.

2.3.7. Harvesting of *L. angustifolius* roots

Infected and/or phosphite-sprayed Lupin roots were harvested by removing two cm of root tips with a scalpel. Root tips were snap frozen, freeze-dried and stored at -80°C until further use. Three biological replicates were taken for each treatment and three root tips were taken for each biological replicate.

2.4 Protein extraction

Freeze-dried mycelia, zoospores and root tissue were ground using metal beads and a tissue mill (Retsch) in liquid nitrogen and an extraction buffer was used to extract and solubilise proteins. The extraction buffer s composed of 25 mM Tris-HCl pH 7.5, 0.25% w/v SDS, 50 mM sodium phosphate, 1 mM sodium fluoride, 50 µm sodium orthovanadate,1 mM PMSF and a protease inhibitor cocktail (Sigma, St Louis, USA). Samples were kept on ice for 30 minutes with regular gentle mixing and centrifuged

at 20,000g at 4°C for 30 minutes. The supernatant containing the protein extract was transferred to a new tube. The isolated secretome was snap frozen, freeze-dried and stored at -80°C until further (Bianco and Perrotta, 2015).

2.5 Protein purification

2.5.1 Purification of sub- proteomes by molecular weight cut-off filters

To purify the proteins from the sub-proteomes by diafiltration (Chapter 3), 3 kDa filters were conditioned with MilliQ water. The mycelial, zoospore and secretome were concentrated and washed twice with MilliQ water. Protein concentration was estimated using the Direct Detect system (Merck Millipore, Darmstadt), snap frozen and freeze-dried until further processing.

2.5.2 Protein purification of sub- proteomes by acetone precipitation

To purify the protein from the sub-proteomes by precipitation (Chapter 4), six volumes of ice-cold acetone were added to the mycelial and zoospore protein extracts. The secretome was resuspended in 3 mL of water and subsequently precipitated with 6 volumes of ice-cold acetone. The samples were incubated at -20 °C overnight and pulse spun to pellet the precipitate. The pellets were washed twice with 1 mL of ice-cold acetone (Crowell et al., 2013).

2.5.3 Protein purification of root tissue by chloroform-methanol precipitation

Lyophilised plant root tissue was ground using metal beads and a tissue mill (Retsch). 300 µl of extraction buffer (125m mM Tris-HCl pH 7.0, 7 % SDS, 0.5 % PVP-40) was added and samples were kept on ice for 30 minutes with regular gentle mixing. The crude extract was centrifuged at 20,000g at 4°C for 30 minutes and 200 µl of the solubilised proteins were decanted. 800 µL of ice-cold methanol and 200 µL of ice-cold chloroform were added to 200 µL of the crude protein extract. Samples were vortexed and 500 µL of water was added. Samples were centrifuged for 5 minutes at 15000g at 4 °C. The upper aqueous phase was removed careful not to disrupt the protein pellet and an additional 500 µL of methanol was added. Samples were inverted and the supernatant was discarded. 1 mL of ice-cold acetone was added and the samples were

incubated at -20 °C overnight. The pellets were washed twice with 1 mL of ice-cold acetone (Wessel and Flügge, 1984).

2.6 Protein digestion and separation

2.6.1 Enzymatic digestion

500 µg of each sample was resuspended in 250 µL 0.5 M triethylammonium bicarbonate (pH 8.5) before reduction and alkylation with 25 µL of 50 mM tris(2-carboxyethyl)phosphine (Thermo Scientific, Waltham) and 12.5 µL 200 mM methyl methanethiosulfonate (Sigma, St Louis) respectively. Samples were digested overnight at 37°C with trypsin (Sigma, St Louis) at a ratio of 1:10, subsequently desalted on a Strata-X 33 µm polymeric reverse phase column (Phenomenex, Torrance, CA, USA) and dried in a vacuum centrifuge (Casey et al., 2017).

2.6.2 High pH reverse phase chromatography

For separation of peptides for proteogenomic work (Chapter 3), dried peptides were separated by high pH reverse phased liquid chromatography on an Agilent 1100 HPLC using a Zorbax Eclipse column (2.1 X 150 mm, 5 um, C18) (Agilent Technologies, Palo Alto). Peptides were eluted with a linear gradient of 20 mM ammonium formate pH 10, and 90 % acetonitrile over 80 minutes. 98 collected fractions were concatenated into 12 fractions and dried in a vacuum centrifuge (Wang et al., 2011; Yang et al., 2012).

2.7 Mass spectrometry

2.7.1 Fractionated qualitative proteomics

For the fractionated peptides (section 2.6.2. High pH reverse phase chromatography) 1 µg from each fraction was loaded onto a Shimadzu Prominence nano HPLC system (Shimadzu, Kyoto, Japan). Peptides were resolved over 180 minutes and eluted with a gradient of 10-40% acetonitrile (0.1% formic acid) into a 5600 TripleTOF mass spectrometer (AB Sciex, Framingham, MA). The data was acquired with the Analyst TF

1.6 software (AB Sciex, Framingham, MA). This data was used for proteogenomics analysis (Chapter 3).

2.7.2 Label-free quantification mass spectrometry

Dried peptides from each sub-proteome, *In vitro* phosphite treated MU94-48 and CPSM366, and all *In planta* protein extractions were not fractionated (Chapters 4 and 5). Peptides were resuspended in 100 µL of 2% acetonitrile and 0.1% formic acid and 1 µg was loaded onto an Acclaim™ PepMap™ 100 C18 LC Column, 2 µm particle size x 150 mm (Thermo Scientific) coupled to a Q Exactive HF mass spectrometer (Thermo Scientific) and separated with a linear gradient over 190 minutes of water/acetonitrile/0.1% formic acid (v/v) (Ali et al., 2014).

2.8 Bioinformatics analysis

2.8.1 Qualitative proteomics

For proteogenomics work (Chapter 3), mass spectra from the mycelia, zoospores and secretome were analysed using Protein Pilot 4.5 Beta Software (July 2012; Sciex). The MS/MS spectra were searched against the genomic proteins (unpublished and publicly available at https://mycocosm.jgi.doe.gov/Phyci1) and a generated 6-frame translation constructed from the genome assembly using EMBOSS: getorf (v6.6) (Rice et al., 2000). The Protein Pilot search parameters were Cys Alkylation: MMTS; Digestion: Trypsin; Instrument: TripleTOF 5600; Bias correction and Background correction tabs checked; ID focus: Biological modifications; Search effort: Thorough; Detected protein threshold [Unused ProtScore (CONF)]: 0.05 (10%); False discovery rate analysis tab checked. For protein identification, only proteins with an unused score of >1.3 (peptides identified with >95% confidence) were used as previously described (Casey et al., 2017).

2.8.2 De novo proteogenomics

Peptides matched to the 6-frame translation assembly from the three sub-proteomes were mapped back to their genomic location. BEDtools was used to classify peptides into the following groups in relation to coding regions of genes (CDS): a) peptides more

than 200 base pairs from CDS, b) peptides within 200 base pairs of CDS regions but do not overlap, c) peptides that overlap CDS, and d) peptides only within CDS boundaries (Quinlan and Hall, 2010). CDS Mapper (version 0.6, 2011, https://sourceforge.net/projects/cdsmapper/) was used to further distinguish the peptides based on their frame in relation to the corresponding CDS features. BLASTp was used to facilitate the manual curation of new and edited genes onto the genome (Bringans et al., 2009).

2.8.3 Qualitative and quantitative label-free proteomics

Proteome Discoverer 2.3 was used for all label-free proteomics (Chapters 4 and 5) was performed using the label-free precursor quantification workflow template (Orsburn, 2021). Mass spectra were matched to the *P. cinnamomi* and/or the *Lupin angustifolius* genomes. Relative quantification was performed using the default precursor ion quantifier. For qualitative identification of proteins, proteins with one or more peptise were used. For quantification, proteins with two or more peptides were used. The FDR was set at <1%. The criteria for differential protein abundance between samples were $p < 0.05$ and a ratio of more or less than 10% (Casey et al., 2017).

2.8.4 WolfpSORT localisation prediction

WolfPSORT is a tool used for predicting protein localisation of sets of protein data based on sorting signals, amino acid composition and functional motifs (Horton et al., 2007). It's classification includes extracellular, cytosol, nuclear, membrane and several other intracellular compartments. To confirm the enrichment of mycelia, zoospores and secretome, the identified proteins of each sub-proteome were analysed using WolfPSORT (version 0.2, plant parameters).

2.8.5 BLASTp for proteogenomic annotation of the genome

For peptides that challenged the CDS features or suggested the presence of unsuspected gene models, BLASTp (version 2.9.0, 2019) homology searches were performed to compare these genes to known *Phytophthora* genes of other species. BLASTp search parameters were as follows; organism: *Phytophthora* (tax ID 4783),

expect threshold: $2E^5$, matrix: PAM30, gap costs: existence 9 and extension 1. All peptides that returned significant scores ($e<1E^{-3}$) were considered for manual annotation. Additionally, tBLASTn (version 2.11, 2020) was used to determine if novel peptides shared any homology across species. To accurately alter gene models, peptides that did not return any significant BLAST results were not used Although these peptides may lead to novel proteins being identified, the manual annotation requires guidance from known genes to assist with sequence features such as frame shifts and spanning introns. Those that returned significant hits were transferred onto the *P. cinnamomi* genome and manually integrated if they complied with sequence features such as start/ stop codons and non-sequenced regions (i.e. regions pf the genome that were not complete).

2.8.6 Interpro, Gene Ontology, KEGG and PFAM

Interpro scan was used to assign putative function to all detected proteins. For the proteogenomics work, Interpro scan (version 79.0) and EGGNOG-Mapper (version 2) was used to generate Interpro, Gene Ontology, KEGG and PFAM assignments for each protein sequence (Zdobnov and Apweiler, 2001; Huerta-Cepas et al., 2017). For all subsequent work, Interpro scan version 86.0 was used to generate Interpro, Gene Ontology and KEGG assignments. PFAM (version3 4.0) was used to generate protein family assignments, EffectorP (version 3.0) was used to predict effector proteins and PHI-BASE (version 4.12) was used to identify significant homology of proteins to known virulence-associated proteins of other species (Winnenburg et al., 2006; Sperschneider et al., 2016; Sperschneider and Dodds, 2022).

Chapter 3 Gene validation and remodelling using proteogenomics of *Phytophthora cinnamomi,* the causal agent of dieback.

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Background

The curation of genes is traditionally accomplished through *in silico* prediction and transcript analysis such as RNAseq. Accuracy of annotations is important in correctly characterising genes and ultimately elucidating their functions. Unlike the transcriptome, the proteome informs on the biochemistry of an organism in real-time. The repertoire and abundance of proteins of an organism in a particular state and time can inform on biological function. Although protein content is derived from the transcriptome, there are discrepancies in the correlation between transcripts and their protein products. Proteogenomics is the integration of protein spectral data to assist in the annotation of genes without the transcript intermediate. Proteogenomic pipelines have been used in other phytopathogens to identify inaccuracies in their genome annotations and facilitate the curation of otherwise unsuspected genes. A draft genome of the phytopathogenic oomycete *Phytophthora cinnamomi* is publicly available (unpublished at https://mycocosm.jgi.doe.gov/Phyci1 by Wayne Reeve, Murdoch University) (Nordberg et al., 2014). To improve the accuracy of this genome for use in subsequent work, a proteogenomic pipeline was applied to correct for errors and curate new gene models.

Methodology

The sub-proteomes mycelia, zoospores and secretome were selected as representatives of the complex life cycle of *P. cinnamomi* and thus capturing a wider spectrum of the total proteome. The sub-proteomes were separated by high pH reverse phase chromatography and analysed by liquid chromatography-mass spectrometry. The mass spectra were matched to both the draft gene annotation and a generated 6-frame translation.

Bioinformatic tools were used to match the spectra to both gene sets and identify discrepancies in the original annotation and subsequently alter the previously annotated genes and manually annotate new gene models.

Result and discussion

Enrichment of the three sub-proteomes was demonstrated by observing their purity microscopically, differences in chromatographic separation and the use of an intracellular marker to determine the amount of intracellular protein contamination in the secretome. Mass spectra matched to 2764 of the predicted proteins from the draft gene annotation, verifying approximately 10% of the total predicted proteome. 19%, 8% and 16% of the total proteins were unique to the mycelia, secretome and zoospores respectively.

Using a proteogenomics pipeline, the coding regions of 44 genes were modified by extension and 14 were altered by merging features. Additionally, this pipeline allowed for the annotation of 23 new gene models. Of these new genes, 21 had predicted functions through PFAM, GO and/or KEGG, which assign functions based on sequence homology. Here I have demonstrated an error rate of approximately 2% in the detected proteome, much of which focuses on defining the boundaries of the coding regions of genes.

Implication

This pipeline can be continually applied to the genome with the acquisition of more spectral data to further improve the accuracy of the annotation. Obtaining spectral data from *P. cinnamomi* in different conditions may be the key to identifying important pathogenicity factors that remain elusive with *in silico* prediction such as effector molecules. There is currently a large research focus on effector identification as they play crucial roles in the process of phytopathogenic infection of plants. Although some can be predicted using *in silico* methods, sequence features and homology are not definitive determinants of these molecules. Therefore, proteogenomic pipelines such as these could assist in the characterisation of such molecules and subsequently be exploited for new and innovative methods of disease control.

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Supporting information

1. For identification, proteins with 1 supporting peptide were used. This methodological choice was made to ensure all potential peptides were identified and cast a wider net to be able to discover new genes. Even with one peptide, the confidence that this peptide was identified correctly was >95% (Bringans et al., 2009). Therefore to ensure all potential matches were included, once peptide matches were sufficient.

2. Although the mycelia and zoospores were produced under different *in vitro* conditions (zoospore production requires a multi-step process), the resulting cells were shown to be purified. This may introduce a confounding variable however there was no experimental alternative to this method and the methods used to produce these cells were optimised to reduce the number of additional confounding variables.

3. GAPDH may not necessarily indicate cell lysis and has been identified in cell walls and may also be secreted. This may explain the low amount of GAPDH that was found in the secretome. However, it was relatively lower than the mycelia and zoospores and sufficient enrichment was achieved.

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ORIGINAL RESEARCH
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Gene Validation and Remodelling Using Proteogenomics of *Phytophthora cinnamomi*, the Causal Agent of Dieback

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Phytophthora cinnamomi is a pathogenic oomycete that causes plant dieback disease across a range of natural ecosystems and in many agriculturally important crops on a global scale. An annotated draft genome sequence is publicly available (JGI Mycocosm) and suggests 26,131 gene models. In this study, soluble mycelial, extracellular (secretome), and zoospore proteins of *P. cinnamomi* were exploited to refine the genome by correcting gene annotations and discovering novel genes. By implementing the diverse set of sub-proteomes into a generated proteogenomics pipeline, we were able to improve the *P. cinnamomi* genome annotation. Liquid chromatography mass spectrometry was used to obtain high confidence peptides with spectral matching to both the annotated genome and a generated 6-frame translation. Two thousand seven hundred sixty-four annotations from the draft genome were confirmed by spectral matching. Using a proteogenomic pipeline, mass spectra were used to edit the *P. cinnamomi* genome and allowed identification of 23 new gene models and 60 edited gene features using high confidence peptides obtained by mass spectrometry, suggesting a rate of incorrect annotations of 3% of the detectable proteome. The novel features were further validated by total peptide support, alongside functional analysis including the use of Gene Ontology and functional domain identification. We demonstrated the use of spectral data in combination with our proteogenomics pipeline can be used to improve the genome annotation of important plant diseases and identify missed genes. This study presents the first use of spectral data to edit and manually annotate an oomycete pathogen.

Keywords: proteogenomics, oomycete, phytophthora, proteomics, dieback

BACKGROUND

The primary role of a genome sequence is to elucidate the entire set of genes expressed by an organism. *In silico* prediction platforms are the main methods for predicting reliable gene sets. However, they can be problematic as transcriptome data does not always correlate with the protein products and their abundance (Wright et al., 2012). Curating genes correctly and

accurately is fundamental in defining the biochemical composition of an organism (Jones et al., 2018). Sequence transcripts and orthologues from related and similar organisms are the primary methods in accurately predicting such genes and identifying interesting and relevant biological components (Mathe, 2002). Evidence-based curation includes transcript data and associated functional annotation such as Gene Ontology (GO) and Protein Families (PFAM) based on sequence homology to other related species (Liang et al., 2009; McDonnell et al., 2018). The challenges in defining comprehensive gene products result in under-represented annotations and incorrectly defined exon boundaries that can miss biologically important features of a genome.

Proteogenomics is a proven but underutilised technology that integrates high confidence peptide data derived from mass spectrometry analysis with genomics as a method to improve gene annotation (Renuse et al., 2011; Nesvizhskii, 2014; Ruggles et al., 2017). Proteogenomic pipelines have been used in phytopathogenic fungi such as *Parastagonospora nodorum*, where mass spectra were used to validate transcriptomic data, edit the genome annotation and identify new candidate genes, generating a more accurate genome that can be used for downstream work (Bringans et al., 2009; Syme et al., 2016). Proteogenomic analysis has also allowed the identification of potential effector molecules in fungi, which has important implications for characterising virulence and understanding the plant-host interface. The proteome of the causal agent of black spot in pear, *Venturia pirina,* was analysed by mass spectrometry and 1,085 novel protein groups were identified, 14 of which were fungal candidate effector genes (Cooke et al., 2014). This provides useful insight into the mechanisms of pathogenicity and has the potential to be exploited to control oomycete and fungal plant pathogens.

Phytophthora cinnamomi is a phytopathogenic oomycete that causes dieback and root rot in natural and agricultural systems across the globe. Its hosts include many species of native Australian flora, as well as crops such as avocado and macadamia (Hardham, 2005). Oomycetes proliferate by releasing motile, asexual units of reproduction, called zoospores. When temperatures and humidity reach favourable levels, *P. cinnamomi* produces fruiting bodies called sporangia which expel free swimming zoospores into the environment, allowing the organism to spread between susceptible hosts. As the zoospores colonise a hosts root system, mature structures including those required for sexual reproduction and nutrient acquisition form, eventually killing the host. The sexual life cycle of *P. cinnamomi* requires two mating strains to produce sexual oospores, which can persist in soil (Crone et al., 2013). Due to the host range, ability to survive harsh environmental conditions and aggressive pathogenicity, *Phytophthora* is recognised as one of the most economically important oomycete genera, with insufficient existing control strategies to minimise its impacts (Hardham, 2005; Kamoun et al., 2015). Despite its economic and ecological importance, little is known about the molecular mechanism of *P. cinnamomi* phytopathogenicity. It is hypothesised that *P. cinnamomi* secretes effectors based on studies on other oomycetes such as *P. infestans* and *P. ramorum* (Birch et al., 2006). Virulence and infection related molecules such as β-cinnamomin have been identified in *P. cinnamomi* (Horta et al., 2010).

A genome sequence of the West Australian *Phytophthora cinnamomi* MU94-48 isolate was established (unpublished and publicly available at https://mycocosm.jgi.doe.gov/Phyci1). This is a valuable tool that can be used to identify effectors and elucidate the molecular mechanisms of virulence (Tyler et al., 2006). The version 1 (V1.0 assembly has a coverage of 69.6x and comprises 9,537 contigs, 1,314 scaffolds with 26,131 predicted gene models. The predicted gene models of *P. cinnamomi* are inflated compared to many *Phytophthora* species such as *P. infestans, P. ramorum, P.capsici*, and the more closely related *P. sojae,* which have reported 17,797, 16,066, 19,805, and 15,743 gene models, respectively (Tyler et al., 2006).

Proteomics data has proven a useful tool to improve the genome annotation of several phytopathogens where high quality mass spectra complemented transcriptomic data and identified potential annotation inaccuracies of exon boundaries and unsuspected gene models. We aimed to use spectral data from several *P. cinnamomi* sub-proteomes to assist in gene calling. These sub-proteomes represent a wide coverage of the *P. cinnamomi* proteome and include a diverse repertoire of soluble proteins. Zoospores characterise the infective life stage and the extracellular proteome is likely to contain proteins related to virulence. These were analysed by 2D LC-MS/MS and resulting spectra were matched to the current gene prediction models. To generate a list of peptides which potentially do not match current models, a 6-frame translation was generated and used for spectral matching. A list of peptides indicating potential altered or novel gene models was generated using the genomic coordinates and 6-frame open reading frames. These were subsequently used to carefully manually edit current annotations and curate novel features on a homology basis with proteins of similar species. Using this proteomics dataset, we refined the genome for downstream proteomic work which will aid the identification of virulence factors and metabolic targets for chemical control. By working toward completing the *P. cinnamomi* genome, downstream proteomic work will be more accurate as the gene set is more representative of what is being expressed. There is also the potential for effector virulence gene discovery and improved biochemical characterisation which can lead to development of resistance (R) gene inclusion in hosts and more targeted methods of chemical control.

Abbreviations: GO, Gene Ontology; PFAM, Protein Families; 1D SDS-PAGE, 1 Dimensional Sodium Dodecyl Suphate Polyachrilamide Gel Electrophoresis; RP-HPLC, Reverse phase High pressure liquid Chromatography; LC-MS, Liquid Chromatography- Mass Spectrometry; MS, Mass Spectrometry; TOF, Time of Flight; CONF, Confidence; FDR, False Discovery Rate; CDS, Coding Sequence; CAI, Codon Adaptation Index; GAPDH, Glyceraldehyde Phosphate Dehydrogenase; JGI, Joint Genome Institute; ORF, Open Reading Frame; KEGG, Kyoto Encyclopaedia of Genes and Genomes; KO, KEGG Orthologues; EC, Enzyme Code.

METHODS

Growth and Maintenance of *P. cinnamomi*

P. cinnamomi MU94-48 (Centre for Phytophthora Science and Management, Murdoch University, Western Australia) stocks

plugs were stored in sterile water in McCartney bottles and grown on V8 agar at room temperature in the dark. For mycelial, secretome, and zoospore production, four plates were used for each biological replicate. Mycelia grown for 4 days were scraped from the plate and were inoculated into Riberio's minimal media supplemented with 25 mM glucose (Ribeiro, 1978). The cells were incubated for 3 days at 24°C in the dark. Mycelia were isolated by centrifugation and the culture filtrate containing secreted proteins was decanted and philtre sterilised. The mycelial pellet was washed twice with MilliQ water and observed microscopically to confirm that hyphal cells predominated. Formation of sporangia and subsequent release of zoospores were produced as previously described (Byrt and Grant, 1979). For the production of zoospores from sporangia, mycelia were grown on V8 agar plates with a 5 × 5 cm sheet of miracloth on the surface and incubated for 4 days at 24°C in the dark. The mycelial mat was transferred into V8 liquid media and incubated on a shaker at 100 rpm for 24 h at 24°C under fluorescent light. The mycelial mat was subsequently washed three times in a sterile solution of 10 mM calcium nitrate, 5 mM potassium nitrate, 5 mM magnesium sulphate and 1:1,000 v:v chelated iron solution and further incubated in this solution on a shaker at 100 rpm for 24 h at 24°C under fluorescent light. The mycelial mat was transferred to a petri dish and incubated for 1.5 h in water at room temperature followed by 30 min at 4°C for the formation of sporangia and release of zoospores. Release of zoospores was observed under the microscope and the zoospore suspension was slowly (to prevent encystment of zoospores) passed through a glass wool syringe to remove any mycelial fragments. Zoospores were harvested by centrifugation at 3,000 g for 30 min, observed microscopically to ensure purity, and counted with a haemocytometer. This incubation process was repeated until sufficient zoospores were harvested. Approximately $4.8E^5$ spores were used for each biological replicate.

Protein Extraction

Mycelia and zoospores were ground using mortar and pestle in liquid nitrogen. An extraction buffer of 25 mM Tris-HCl pH 7.5, 0.25% SDS, 50 mM sodium phosphate, 1 mM sodium fluoride, 50 μM sodium orthovandate, and 1 mM phenylmethalsulphonym F all in the presence of a protease inhibitor cocktail (Sigma, St Louis) was used to extract and solubilise proteins as previously described (Resjö et al., 2014). Samples were kept on ice for 30 min with regular gentle mixing and centrifuged at 20,000 g at 4°C for 30 min. The protein solutions were subsequently desalted and protein amount was estimated using Direct Detect cards (Merck Millipore, Darmstadt). Mycelial, secretome, and zoospore extractions yielded ∼1.4, 0.6, and 0.7 mg of protein, respectively. All samples were freeze dried before further processing. SDS-PAGE was performed for all samples to ensure proteolysis was minimal.

Sample Preparation

To visualise each sub-proteome, 20 μg of each sample was loaded onto a 1D SDS-PAGE. To determine the amount of intracellular contamination in the extracellular proteome, the activity of an intracellular enzyme marker glyceraldehyde phosphate dehydrogenase (GAPDH) was assayed on each sub-proteome as per the manufacturer's instructions (Sigma, St Louis). Five hundred microgram of each sample was resuspended in 250 uL 0.5 M triethylammonium bicarbonate (pH 8.5) before reduction and alkylation with 25 uL of 50 mM tris (2-carboxyethyl)phosphine (Thermo Scientific, Waltham) and 12.5 uL 200 mM methyl methanethiosulfonate (Sigma, St Louis), respectively. Samples were digested overnight at 37°C with trypsin (Sigma, St Louis) at a ratio of 1:10, subsequently desalted on a Strata-X 33 um polymeric reverse phase column (Phenomenex, Torrance, CA, USA) and dried in a vacuum centrifuge.

High pH Reverse Phase Chromatography

Dried peptides were separated based on hydrophobicity by high pH reverse phased liquid chromatography on an Agilent 1100 HPLC system using a Zorbax Eclipse column (2.1 × 150 mm, 5 μm, C18) (Agilent Technologies, Palo Alto) (Siu et al., 2011; Zhang et al., 2014). Peptides were eluted with a linear gradient of 20 mM ammonium formate pH 10, 90% acetonitrile over 80 min. A total of 98 fractions were collected, concatenated into 12 fractions based on collection order and dried in a vacuum centrifuge. The UV trace was used to visualise the total peptide content and depth of each sub-proteome.

Nano LCMS/MS

Fractions were resuspended in 100 uL of 2% acetonitrile and 0.1% formic acid and loaded onto a Shimadzu Prominence nano HPLC system (Shimadzu, Kyoto, Japan). Peptides were resolved with a gradient of 10–40% acetonitrile (0.1% formic acid) at 300 nL/min over 180 min and eluted through a nanospray interface into a 5600 TripleTOF mass spectrometer (AB Sciex, Framingham, MA). The data was acquired in an information-dependent acquisition mode with Analyst TF 1.6 software (AB Sciex, Framingham, MA). The MS settings were as follows: Ionspray Voltage Floating = 2,300 V, curtain gas = 20, ion source gas 1 = 20, interface heater temperature = 150, and declustering potential = 70 V. The TOF MS scan was performed in the mass range of 400–1,250 Da with a 0.25 s TOF MS accumulation time, whereas the MS/MS product ion scan was performed in the mass range of 100–1,800 Da with a 0.1 s accumulation time. The criteria for product ion fragmentation were set as follows: ions (>400 and <1,250 *m/z*) with charge states of between 2 and 5 and an abundance threshold of >250 cps. Former target ions were excluded for 10 s after one occurrence. The maximum number of candidate ions per cycle was 20.

Data Analysis

Mass spectral data were analysed using Protein Pilot 4.5 Beta Software (July 2012; Sciex). MS/MS spectra were searched against the genomic proteins and the 6-frame translated data set constructed from the genomic assembly scaffolds using EMBOSS: getorf (v6.6). Search parameters were: Cys Alkylation: MMTS; Digestion: Trypsin; Instrument: TripleTOF 5600; Special factors: None; Quantitation tab checked: Bias correction and Background correction tabs checked; ID focus: Biological modifications;

Search effort: Thorough; Detected protein threshold [Unused ProtScore (CONF)]: 0.05 (10%); False discovery rate analysis tab checked. All identified proteins had an Unused Protscore of >1.3 (peptides identified with >95% confidence), as calculated by the software and a global false discovery rate of <0.1% determined at the protein level. To determine the sub-proteome enrichment, the resulting sequences of matched proteins were analysed using the protein localisation tool WolfPSORT (version 0.2, plant parameters) (Horton et al., 2007). Proteins were assigned to a predicted sub-cellular location based on sorting signals, amino acid composition, and functional motifs.

De novo Proteogenomics

Peptide matches to the 6-frame translated assembly from the sub-proteomes were combined and mapped back to their genomic location and a set of criteria described below were applied to determine which genes suggest incorrect boundary annotations and which peptides support discovery of new genes. Firstly, BEDtools (version 2.28.0, 2019) was used to distinguish peptides into the following groups using the intersect and subtract features: (a) peptides more than 200 base pairs from coding regions of genes (CDS), (b) peptides within 200 base pairs from CDS features but do not overlap CDS boundaries, (c) Peptides that overlap CDS boundaries, and (d) peptides that remain within CDS feature boundaries (Bringans et al., 2009; Quinlan and Hall, 2010). Subsequently, the CDS Mapper tool (version 0.6, 2011, https://sourceforge.net/projects/cdsmapper/) was used with default parameters to further classify these based on their frame match to corresponding CDS features of the annotated draft genome (Bringans et al., 2009). All peptides suggesting novel or altered gene models were blasted (BLASTp, version 2.9.0, 2019) with the following search parameters: organism: *Phytophthora* (tax ID 4783), expect threshold: $2E^5$, word size: 2, matrix: PAM30, gap costs: existence 9 and extension 1. All peptides with significant returns ($e<1E^{-3}$) were considered for manual annotation. An additional blast search using tBLASTn (version 2.11, 2020) was also used to indicate if novel (unannotated) sequences might exist that are shared among *Phytopththora* species. Peptides that did not return significant results were not used for this analysis. All significant BLAST hits were transferred onto the *Phytophthora* draft genome and manually edited to comply with sequence features such as start/stop codons and non-sequenced regions. The novel annotated genes were further analysed for total number of supporting peptides (as per Protein Pilot methods described above). Genes that had only one high confidence peptide were included for the purposes of gene discovery (Sheng et al., 2012). Protein Family domains (PFAM), Gene Ontology (GO), terms and Kyoto Encyclopaedia of Genes and Genomes (KEGG) were also assigned using Interpro scan (version 5.44-79.0, 2020) using and EGGNOG-mapper (version 2, 2019) using default parameters. To determine whether any pathogenesis related proteins were present within the novel set, annotations were analysed for presence of any potential virulence factors using PHI-BASE (version 4.9, 2020) using default parameters (Urban et al., 2017). The Codon Adaptation Indexes (CAIs) of each novel gene were also calculated using Emboss CAI (version 6.6, default parameters), which indicated gene annotation with anomalous usage of codons (Sharp and Li, 1987).

RESULTS

Sub-proteome Enrichment

To obtain a representative proteome of *P. cinnamomi*, vegetative mycelia, and transient short-lived zoospores of *P. cinnamomi* were used as these are the dominant cell types that grow and initiate infection in hosts. In addition, we extracted soluble secreted proteins (secretome) from the mycelia, which are widely studied due to their implications on pathogen-host interactions. The purity of the mycelia and zoospores was observed under a stereoscope (**Figure 1**). **Figure 1A** shows no evidence of intercellular contamination and demonstrated the purity of these cell types. The large mass of mycelia had not produced zoospores or their precursor (the sporangia) in this method of *in vitro* cell culture. Similarly, vegetative mycelia was not observed in the zoospore preparation (**Figure 1B**).

1D SDS-PAGE was run to visualise the sub-proteomes of each cell type (**Figure 2A**). The banding patterns of each sub-proteome show differences in total protein content. The extracellular proteome showed enrichment in lower molecular weight proteins whereas the mycelia and zoospores had proteins that spanned over the whole mass range. To test the purity of the secretome, an enzyme activity assay of the cytoplasmic marker GAPDH was measured, which should only be present in small amounts (**Figure 2B**) (Tristan et al., 2011). Both the mycelia and zoospores had similar detected amounts of GAPDH detected, ∼4.7 and 4.8 mU/mg protein, respectively. GAPDH was also detected in the secretome, however at lower amounts (1.6 mU/mg protein). The RP-HPLC UV total ion count traces indicated differing protein content between the three sub-proteomes, as majority of the peaks do not match in intensity and retention time (**Figure 2C**). The majority of proteins detected in the mycelial and zoospore were localised intracellularly at 45 and 41%, respectively as predicted by WolfPSORT (**Table 1**). The secretome was enriched in extracellular localisation proteins with a predicted 18% compared to 5% in both the mycelia and zoospores.

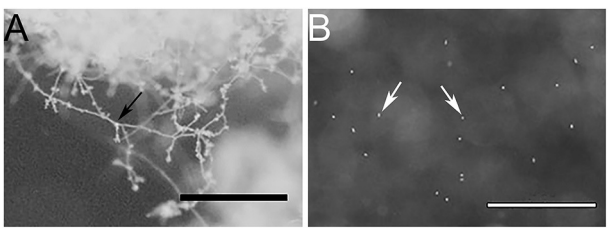

FIGURE 1 | Stereo microscope images of **(A)** a mycelial mass, and **(B)** free swimming zoospores, indicating minimal to no cross contamination between cell types. Bars represent 1 mm scale. Black arrow indicates mycelia and white arrows indicate zoospores.

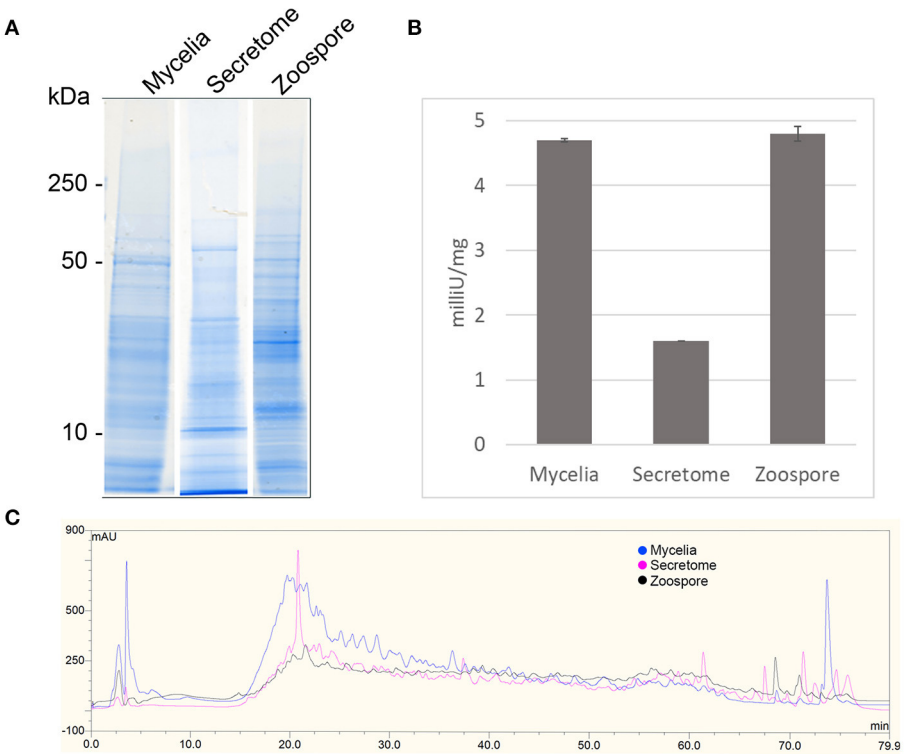

FIGURE 2 | Quality control of sub-proteomes. **(A)** SDS-PAGE analysis of *P. cinnamomi* sub-proteomes. **(B)** Glyceraldehyde phosphate dehydrogenase activity within each sub-proteome indicating relative levels of contamination of intracellular proteins in the extracellular proteome. **(C)** 214 nm UV High pH reverse phase HPLC separation of 500 μg of each sub-proteome demonstrating sufficient peptide separation and differing protein content.

TABLE 1 | WolfPSORT localisation prediction of the whole genome annotation and sub-proteomes.

WolfP SORT	Total no. proteins	Predicted Intracellular	Predicted Extracellular	Low confidence prediction
Whole genome prediction	26,131	43%	6%	51%
Mycelial proteome	3,209	46%	5%	49%
Secretome proteome	1,605	42%	18%	40%
Zoospore proteome	2,304	50%	5%	45%

Validation of V1.0 Gene Models Using Sub-proteome Spectra

The mass spectra were used to validate the draft annotation of the *P. cinnamomi* genome. The annotations acquired from JGI Mycocosm (assembly annotation version 1.0) were designated in this study as "V1.0" and the annotation set containing subsequently manually edited loci was designated "V2.0.0."

Non-redundant peptide matches (at least two 95% confident peptides) resulted in 2,554, 1,362, and 2,304 proteins from the mycelia, secretome and zoospores, respectively. From this data, 2,764 unique proteins from the V1.0 predicted gene set were identified (**Figure 3A**). 526, 215, and 432 proteins were unique to the mycelia, secretome and zoospores, respectively, which implies a wide range of the whole proteome detected. The mycelia and zoospores had more unique protein identifications than the secretome, which may be a result of an expected lower mass range of an extracellular proteome that were below the acquisition detection limits. Additionally, PFAM assignment of the proteins identified in the sub-proteomes, showed differences in composition of domains (**Figure 3B**; **Supplementary Material 1**). Analysis of the top 10 most abundant PFAM of mycelia and zoospores displayed notable differences. The mycelia have unique PFAM domains, including pleckstrin homology (PH) and ATPase associated with diverse cellular activities domain (AAA). The zoospores have unique C2, RCC1, and TIG domains compared to the mycelia and the secretome. When matched to 4,874,027 generated open reading frames (ORF) of the 6-frame translation, 2,752, 1,355, and 2,334 ORFs from the mycelia, secretome and zoospores were identified (**Table 2**). Although this does allow us to match more peptides to the genome than the V1.0 annotation, some level of redundancy is expected from matching to reading frames

that do not form genes. The false discovery rate for all mass spectra analysis was <0.1% using the Protein Pilot decoy database method, which is within the limits of the general consensus for large scale proteomic data (Lam et al., 2010; Bantscheff et al., 2012). Of the V1.0 detected by mass spectrometry, 2,398 had additional support by assigned GO terms and/or PFAM domain.

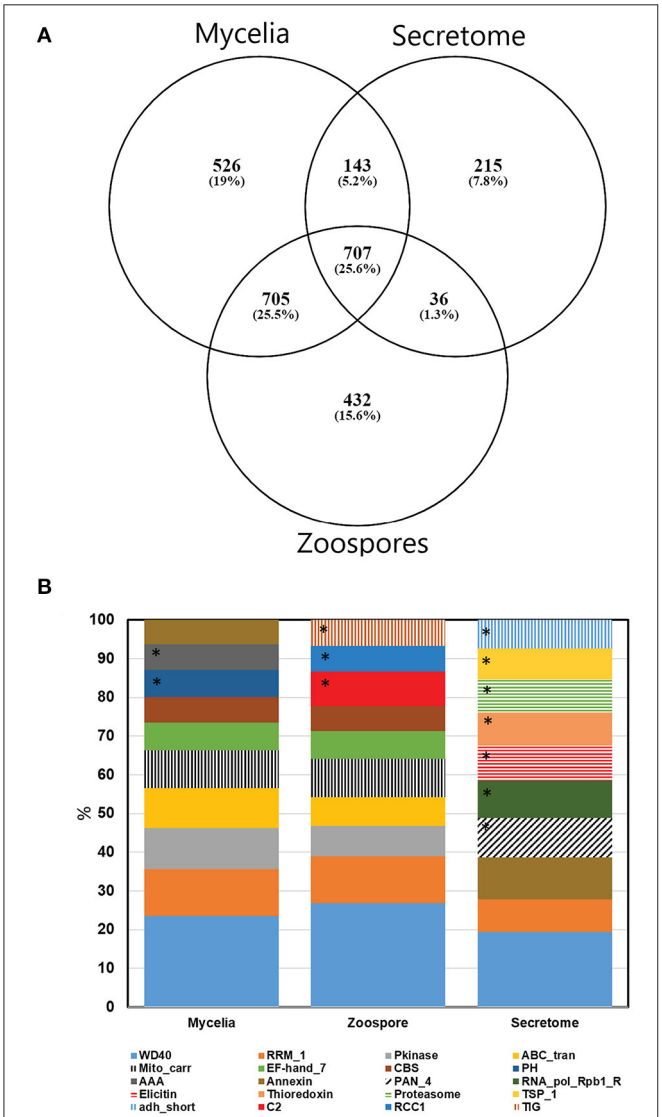

FIGURE 3 | (A) Number of proteins detected by mass spectrometry of each sub-proteome supported by at least two 95% confident peptides. **(B)** Top 10 PFAM domains based on the number of proteins identified in each sub-proteomes. *Represent unique domains to each sub-proteome. For a complete PFAM assignment, refer to **Supplementary Material 1**.

Annotating New Gene Models by Homology Criteria

Although there is peptide support for a large number of the V1.0 genes, it is expected that there are some forms of incorrect intron and exon boundary annotations that can be detected using spectral data. In addition, this spectral data can also be used in the detection of new genes. Twenty-three thousand four hundred fifty-seven unique high confidence peptides matched to the 6-frame ORFs were mapped back to their genomic location. Twenty-two thousand four hundred forty-three peptides mapped completely within coding exon boundaries. Two hundred seventy-four peptides mapped

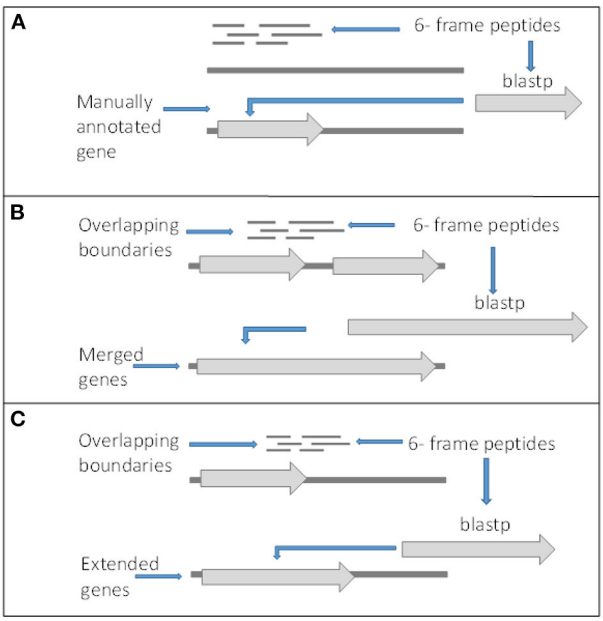

FIGURE 4 | Criteria for gene editing and discovery. **(A)** Peptides matching to 6-frame open reading frames in loci with no surrounding genes or out of frame from surrounding genes and returned significant BLASTp hits were manually annotated as new genes. **(B)** Peptides overlapping boundaries of multiple genes within the same frame and returned significant BLASTp hits were used to manually merged genes. **(C)** Genes with in frame peptides overlapping boundaries and returned significant BLASTp hits were manually edited.

TABLE 2 | Summary of mass spectra identification using the annotated protein and 6-frame open reading frame databases.

Sub-proteome	Total spectra	Database type	Proteins identified (inc. single peptide hits)	% total spectra identified
Mycelia	964,781	Annotated prediction	2,554	36.1
		6-frame translation	2,752	28.6
Secretome	877,718	Annotated prediction	1,362	18.6
		6-frame translation	1,355	11.4
Zoospore	837,256	Annotated prediction	2,304	31.0
		6-frame translation	2,334	22.8

partly within exons (i.e., span across boundaries) and 287 within 200 bp of boundaries (**Figures 4B,C**). Four hundred fifty-three peptides mapped more than 200 bp from exon boundaries (**Figure 4A**). Furthermore, the frame test applied more stringent criteria for frame matching of these peptides to corresponding V1.0 annotations (**Table 3**). A total of 1,010 peptides did not match the frame of corresponding CDS features or were further than 200 bp from any gene models. This suggested 438 gene features with potentially incorrect boundaries. These were considered as candidates for new gene models.

To select peptide candidates that would likely result in alteration of V1.0 genes and curation of new genes, Blastp was used. Peptides that returned significant hits to other *Phytophthora* species were used to manually edit and curate new genes (**Table 4**). This largely reduced the number of potential edited and new genes due to both the redundancies of 6-frame peptides and rigorous Blastp parameters used for peptide matches. Of those with conflicting boundaries, 72 peptides showed significant homology to other *Phytophthora* species. Of the peptides that were further than 200 bp from any gene, a total of 160 peptides returned significant BLASTp hits, suggesting the presence of previously unannotated genes on the *P. cinnamomi* genome. The homologous sequences were transferred onto the *P. cinnamomi* genome and the annotations were manually integrated, taking into consideration differences in the genome and features such as introns. Of the peptides that returned significant hits using the tBLASTn search, 34 did not have a corresponding BLASTp result, suggesting 33 potential new genes across four *Phytophthora* species. However, to annotate new genes, we chose those peptides that were confirmed by both BLASTp and tBLASTn hits to increase confidence. To ensure high confidence of newly annotated genes, we chose those genes that have homologues in other *Phytophthora* species.

Using these criteria, a total of 60 genes were edited, which equates to an error rate of ∼2% of the detected proteome. Of these, 44 were modified by extending the exon boundaries and there were 16 instances of merged genes (**Supplementary Material 2**). Additionally, 23 new previously

TABLE 3 | Confirmation of genes supported by peptides within or crossing exon boundaries.

Total number of genes	**26,478**
Genes confirmed by spectral matching (Protein Pilot)	3,468
Genes with 6-frame peptide support- with boundary conflicts	52
Genes with no 6-frame peptide support (inc. with boundary conflicts)	19,724
Genes with only mismatched 6-frame peptides	69
Genes with matched and mismatched 6-frame peptides	24

TABLE 5 | Summary of original predicted and newly annotated/ edited genes using proteogenomics.

	No. genes
Version 1 prediction	2,6131
Total modified genes	60
Modified by extension	44
Modified by merging genes	16
New gene annotations	23
Total number of version 2 genes	26,151

TABLE 6 | Summary of new gene validation using supporting peptides, PFAM, and Gene Ontology terms using Protein Pilot.

	Merged annotations	**Extended annotations**
Supported by only one peptide	6	8
Supported by two or more peptides	10	36
Genes with PFAM domains	13	39
Edited genes with differing PFAM to V1.0	3	3
Genes with GO terms	13	35
Edited genes with differing GO to V1.0	1	1
Genes with KO assignment	13	34
V2.0.0 genes with differing KO to V1.0	0	1

TABLE 4 | Summary of frame matches of peptides with nearby CDS features and number of significant Blastp and tBlastn hits (E value $> 1E^{-3}$) and number of gene features manually.

	Frame match (CDS)	**Frame mismatch (CDS)**	**Total peptides**	**No. significant BLASTp hits of conflicting peptides**	**No. significant tBLASTn hits matching BLASTp hits**	**No. significant tBLASTn hits with no BLASTp result**
Peptides within CDS boundaries	22,134 (3,178)	309 (242)	22,443	72	49	8
Peptides crossing CDS boundaries	240 (171)	34 (31)	274	157	91	11
Peptides within 200 bp from CDS boundaries	73 (52)	214 (165)	287	109	67	7
Peptides more than 200 bp from CDS features	–	–	453	160	71	8

undefined genes were annotated (**Table 5**). These annotations were uploaded to the GenBank under accessions MT820663-MT820655. The edited annotations will be referred to by original annotation identification with "V2.0.0" suffixed, as listed in the **Supplementary Materials 3, 4**, respectively. In summary, we identified errors in 60 V1.0 genes which were manually altered and added a further 23 annotations to the gene set of *P. cinnamomi*.

Validating Edited and New Genes

The edited genes were subsequently analysed for total peptide support and differences in functional assignment compared to the original annotation. Peptides within the edited regions were manually counted (**Table 6**). Of the extended genes, only one had no other supporting information other than the support of one 95% confident peptide in the extended portion of the gene (e_gw1.28.366.1_V2.0). All other extended genes had support from more than two high confidence peptides and/or homologous functional assignment. Similarly, only one merged gene had a single peptide supporting the merged region of the annotation (gw1.160.19.1_V2.0). All others were supported by two or more high confidence peptides, which is the general requirement for protein identification in proteomics (Bringans et al., 2017). Genes were analysed for GO terms, PFAM domains, and KEGG orthologues (KO) to determine whether the altered boundaries change their functional annotation assignment (**Table 6**). Details of each functional annotation are shown in **Supplementary Materials 3, 4**.

The original mass spectra were matched to the set of new genes (using Protein Pilot- see methods) to determine how many peptides supported each gene (i.e., determine if any genes were a product of single peptide matches) (**Table 7**). Of the 23 new genes identified, one new gene had support from only one high confidence peptide (MT820633). All new genes were detected in the mycelia and most were also identified in the secretome and zoospore (**Supplementary Material 5**). The remaining 22 genes had at least two or more supporting peptides.

To further support this new gene set, protein sequences were analysed for protein function by assignment of PFAM **Supplementary Material 5**. The new annotations were analysed for virulence factors using PHI-BASE. None of these annotations returned a significant hit to any known virulence factors.

TABLE 7 | Summary of new gene validation using supporting peptides, Protein Families, and Gene Ontology terms.

	Number of new gene models
Supported by only one peptide	1
Supported by two or more peptides	22
Contain PFAM domains	21
Contain GO terms	21
Containing KO	17
No functional support	2

*Details of annotations of each entry are shown in **Supplementary Material 5**.*

Codon Adaptation Index

The codon adaptation indices were calculated for the set of new features and compared to the V1.0 gene set to identify significant differences in codon usage and distribution that could indicate possible causes for errors and missed genes (**Figure 5**). The distribution of the CAIs of the new set were significantly different (t-test, $p < 0.05$) than those of the predicted gene set

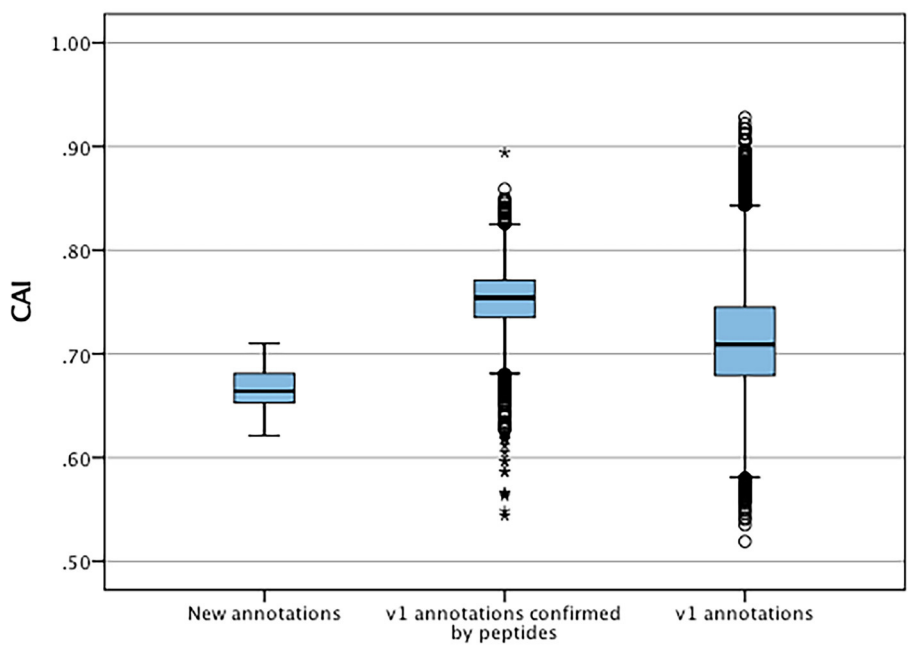

FIGURE 5 | Distribution of codon adaptation indices between new annotation and V1.0 annotations confirmed by mass spectrometry and all V1.0 annotations. *Represent significant differences ($p < 0.05$) of data sets to V1.0 annotations.

TABLE 8 | Sequence feature summary of the new gene set.

	No. features
Total features	23
Start and stop codon present	22
Stop codon only (no start codon)	1

suggesting a higher proportion of less common codon usage in the new set. These were also significantly lower to the CAIs of all original annotations that had high confidence supporting peptides. Each new gene was also checked for the presence of start codons other than methionine and stop codons (**Table 8**). Only one new annotation MT820649 had non-standard codon usage, where an alternate start codon was used in accordance to its homologues in *P. infestans* and *P. sojae*.

DISCUSSION

The three sub-proteomes (mycelia, secretome, and zoospores) represent a diverse range of proteins and capture the majority of the *P. cinnamomi* proteome. Although microscopic observation indicated successful purity in the mycelia and zoospores, the GAPDH assay showed some cytoplasmic contamination in the secretome. Cytoplasmic markers such as GAPDH and malate dehydrogenase have been used in other studies as indicators of intracellular contamination in secretome samples of other organisms (Alexandersson et al., 2013; Kim et al., 2013). In these cases, some level of contamination was similarly observed in isolated secretome samples, likely due to some level of cellular leakage. Other known cytoplasmic markers such as malate dehydrogenase have been observed in fungal secretomes, where their extracellular functions are not known (Tan et al., 2009). This set of enrichment data confirms that the sub-proteomes were sufficiently enriched for this study and the total proteome is diverse and represents both growing and infective stages of development.

The aim of using these three sub-proteomes was firstly to validate as much as the *P. cinnamomi* draft gene set as possible. Through spectral matching, we verified 10.6% of the predicted gene set. The differing sub-proteomes, as shown above, are reflected in the validation of V1.0 genes by spectral matching. Unique protein identifications of ∼19, 8, and 16% of the mycelia, secretome and zoospores, respectively, accounted for the differences in observed SDS-PAGE banding patterns and RP-HPLC traces. Proteomic studies of other *Phytophthora* species indicated variable numbers of unique proteins to these sub-proteomes. A 2- dimensional proteomic study of the oomycete *P. palmivora* indicated 1% unique proteins for mycelia and zoospores (Shepherd et al., 2003). However, a profile of the *P. infestans* secretome indicated similar coverage of extracellular proteins to this study (Meijer et al., 2014). PFAM domain assignment of the sub-proteomes also indicated biochemical distinction. The domains unique to the mycelia are involved in a wide range of intracellular signalling of the vegetative state. The domains distinct in zoospores included C2, RCC1, and TIG. Members of the C2 domain target proteins to cell membranes in response to external stimuli (Farah and Sossin, 2012). In zoospores, this is possibly involved in the process of response to chemotactic gradients in the external environment that control their direction of movement. RCC1 domains include proteins involved in chromosome condensation during transition of cell growth, which could be involved in the quick differentiation from zoospores to cysts (Ohtsubo et al., 1989). Plexins are major constituents of the TIG domain that play roles in signalling of axon growth and may be involved in zoospore motility (Negishi et al., 2005). There are several distinct PFAM domains in the secretome compared to the mycelia and zoospores. Elicitins are commonly found in the secretome of *Phytophthora* species. These molecules induce a hypersensitive response in hosts (Horta et al., 2010). Thioredoxin and proteasome domains were also distinct in this sub-proteome, which are involved in defence to oxidative stress and protein degradation, respectively (Meijer et al., 2014). These are important aspects of the secretome that contribute to the early stages of infection.

Mass spectrometry based proteomics can be used to overcome some of the constraints of traditional gene prediction methods and with continuing advances in proteomics technologies becomes a desirable tool to elucidate the biochemistry of an organism (Cox and Mann, 2007). High throughput proteomic pipelines such as liquid chromatography mass spectrometry (LC-MS/MS) are becoming more sensitive, rapid and less expensive (Law and Lim, 2013). As an added benefit to transcriptome work, quantitative proteomics can inform on differential expression of proteins (Pastorelli et al., 2006).

Using the mass spectra matched to the 6-frame translation of the draft genome, we refined the draft genome of *P. cinnamomi*. Total peptide support and functional assignment were used to validate and to obtain the most accurate representation of edited and newly curated genes. We compared the functional assignments of V1.0 and V2.0.0 edited genes to identify differences inferred by the changes in annotation features. The PFAM domains associated with the extended genes that differed from V1.0 genes were involved in energy production and one recombination protein (gw1.193.42.1_V2.0 and fgenesh1_kg.277_#_5_V2.0). Similarly, the only differing GO was that of DNA repair (CE70043_1777_V2.0). The KO of V2.0.0 extended genes remained mostly the same with minor changes and one enzyme code, an enzyme involved in carbohydrate metabolism was not present in V1.0 genes. These changes include a PAMP recognition signalling factor in gw1.44.72.1_V2.0.0. The majority of merged gene features were mostly between CDS features of the same gene. Therefore, there were minimal functional assignment differences between V1.0 genes and those altered by merging. However, three instances merged whole genes (fgenesh1_kg.79_#_14_#_15_V2.0, estExt_fgenesh1_pm.C_90019, fgenesh1_pm.9_#_20, e_gw1.9.526.1_V2.0 and gw1.243.65.1, gw1.243.79.1_V2.0). fgenesh1_kg.79_#_14_#_15_V2.0 combined two whole genes from the V1.0 annotation, the V2.0.0 functional assignment included an additional PFAM domain, PF12698, an ABC-2 family transporter protein, which are often highly expressed in plant pathogens such as the oomycetes as

they play roles in the biotrophic phase of infection and pathogenicity (Seidl et al., 2011; Ah-Fong et al., 2017). The second, estExt_fgenesh1_pm.C_90019, fgenesh1_pm.9_#_20, e_gw1.9.526.1_V2.0, merged three whole genes, and included three different PFAM domains, two Poly (ADP-ribose polymerase domains and one WGR domain. This edit also resulted in one gene ontology difference, the presence of an NAD+ ADP-ribosyltransferase. There were no other differences in the GO and KEGG ontologies between V1.0 and V2.0.0 merged genes. Although these functional differences do not indicate major functional differences, they can impact the way in which we classify these proteins when trying to understand their role in a system.

The newly curated genes were validated using total peptide support, functional assignment and also examined for their codon usage to gain a better understanding of why they were missed in the V1.0 annotation. Although MT820633 had only one supporting peptide, it had PFAM, GO and KEGG assignment, all indicating its function to be associated with ankyrin, a protein family that is involved in the formation of the cytoskeleton and has been associated with signal transduction in other oomycete pathogens (Torto-Alalibo et al., 2005). MT820636, MT820637 and MT820651 although had significant blast hits to other oomycetes but did not have any PFAM, GO, or KO assignments. Functional domains were identified for all other new genes, most of which were related to general biochemical processes, including energy production, translation and transporter activity. PFAM domains and GO assignments that were associated with new genes but not present in V1.0 genes were mostly domains of ribosomal proteins and one ferredoxin type domain. Ribosomal proteins are highly conserved between species of oomycetes. This has been shown in *Pythium insidiosum* using expressed sequence tags, that show homology between several of the *Phytophthora* species (Win et al., 2006; Krajaejun et al., 2011). Ferredoxin domains have been identified in *P. parasitica* and were found to be associated with ATP generation (Le Berre et al., 2008). Of the KO assignments, nine from V2.0.0 genes were not present in V1.0 genes. These were mostly associated with general metabolic and cellular functions, translation and genetic information processing functions, with many domains associated with ribosomes (**Supplementary Material 5**). The remaining peptides matched to the 6-frame translation that were suggestive of 34 new genes but did not return significant BLASTp results to genes of any other *Phytophthora* species. These 34 hits are still potentially interesting new genes but would need to be validated using other means such as rapid amplification of cDNA ends on the *P. cinnamomi* and other *Phytophthora* species genomes.

Effector proteins in *Phytophthora* species often contain characteristic motifs such as those conserved in RXLR, Crinkler and necrosis inducing *Phytophthora* protein families (Meijer et al., 2014). These effectors can translocate into host cells and manipulate host immunity in order to successfully colonise hosts (Wang and Jiao, 2019). Characterising effectors is important to understand the mechanisms of pathogen infection and can subsequently be exploited for plant protection. This can be achieved through the development of resistant hosts through stacking of R genes that recognises these effectors, thereby inducing a defence response (Vleeshouwers and Oliver, 2014). Effector prediction is problematic as not all of these molecules have the characteristic motifs (Sperschneider et al., 2015). But the prediction of candidate effectors can be maximised with the use of a combination of tools (Sonah et al., 2016). The present study was unable to detect any candidates that are homologous to characterised effectors using PHI-BASE, showing no significant homology to virulence factors in the new gene set. However, of the total identified proteins in V1.0 genes, putative elicitins with homology to INF1 were identified in the secretome. Elicitins can be recognised as pathogen associated molecular patterns or by corresponding R genes to induce a hypersensitive response in hosts (Horta et al., 2010). Effector discovery *in vitro* can be difficult as there is minimal stimulation to produce and release virulence factors such as effector molecules. Typically, studies aiming to identify virulence factors such as effectors simulate a host interaction environment as plant pathogens primarily express these molecules at early stages of infection to overcome host defence systems (Baldwin et al., 2006; Bozkurt et al., 2012; Urban et al., 2017). This data also complies with the GO, PFAM, and EC assignments, as the majority of functional annotations indicated core metabolic functions and therefore are unlikely to have virulence or infection related functions.

The significantly lower codon adaptation indices of the new genes compared to detected V1.0 genes can suggest a limited rate of protein translation, which implies that over time optimised transcriptional levels have a selective advantage for gene expression. This can also be influenced by repeat- induced point mutations which can have implication on codon frequencies, and ultimately CAIs (Testa et al., 2016). Additionally, recent lateral gene transfers can result in altered codon frequencies as these involve acquiring genes that have codons optimised for different species (Tuller, 2011). We also observed a bias of CAIs in V1.0 annotations confirmed by mass spectrometry compared to the CAIs of all V1.0 genes. These experimentally confirmed genes had higher CAIs, which indicates that the highly abundant proteins sampled in this study are translated with high efficiency. The only new gene without a typical AUG start codon was MT820649, which is a high confidence prediction with evidence from seven supporting peptides. BlastP analysis of MT820649 revealed high homology with sequence ribosomal protein orthologues found in other well-annotated *Phytophthora* species such as *P. sojae* (**Supplementary Material 5**). These orthologues also lacked the typical AUG start codon. Upon close inspection of the upstream nucleotide sequences of MT820649 and its orthologue in *P. sojae*, we observed evidence of typical eukaryotic alternate CUG start codon that encode leucine (**Supplementary Material 5**) (Starck et al., 2012). The usage of alternate start codons were frequently observed in other eukaryotic organisms. In most cases, alternate start codons differ from AUG by a single nucleotide (Kearse and Wilusz, 2017). The CUG leucine codon is a typical alternate start codon observed as an initiator of translation in major histone compatibility complex in mammals (Starck et al., 2012; Sendoel et al., 2017). As such, we are currently using rapid amplification of cDNA ends approach to validate the 5′ terminal transcript sequence of MT820649 and its

orthologues in other *Phytophthora* species (Yeku and Frohman, 2011). The remaining 28 novel predicted genes possessed regular start and stop codons as expected.

CONCLUSION

The data generated by shotgun LC-MS/MS confirmed 2,764 previously annotated gene models from the *P. cinnamomi* draft genome using high quality mass spectra from a diverse range of sub-proteome fractions. The spectral data suggested potential errors in gene calling, and using the spectral data, we were able to alter 60 genes by extending and merging exons, and identify 23 previously undescribed annotations in the *P. cinnamomi* genome. This demonstrates that the correlation between genes called by methods *in silico* are not always correlated to protein products, with evidence of annotation error rates of 2% of the detected proteome. This work demonstrates there are effective ways to use proteomics to correct boundary discrepancies and discover new genes. To our knowledge, this study presents the first use of spectral data to edit and manually annotate an oomycete pathogen. As more spectral data is accumulated, we expect there will be additional changes to the annotation including the discovery of more new genes.

DATA AVAILABILITY STATEMENT

Spectral data used for this study is available at Dryad (doi: 10.5061/dryad.7h44j0zsc). Newly curated genes have been submitted to Genbank under the accessions MT820633-MT820655.

AUTHOR CONTRIBUTIONS

CA and K-CT conceived and designed the study. CA performed all the experiments and prepared the draft of the manuscript. CA and JH performed the bioinformatics analysis. GH and SJ provided intellectual input into the study. SB and RL provided input into proteomics sample preparation and mass spectrometry analysis. All authors revised and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.665396/full#supplementary-material

Supplementary Material 1 | PFAM domain assignment of proteins identified in each sub-proteome.

Supplementary Material 2 | CDS coordinates of edited genes, Description: Gene coordinates of manually edited V1.0 genes. Gene identification names include the original ID from https://mycocosm.jgi.doe.gov/Phyci1 as a reference.

Supplementary Material 3 | Master table of genes edited by extending features, Description: Summary of sequence, sequence features, peptide support, codon usage and functional assignment of edited genes generated by extending features.

Supplementary Material 4 | Master table of genes edited by merging features, Description: Summary of sequence, sequence features, peptide support, codon usage, and functional assignment of edited genes generated by merging features.

Supplementary Material 5 | Master table of new genes, Description: Summary of sequence, sequence features, peptide support, codon usage, and functional assignment of new genes.

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Chapter 4 Comparative sub-cellular proteome analyses reveal metabolic differentiation and production of effector-like molecules in the dieback phytopathogen *Phytophthora cinnamomi.*

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Background

The polycyclic life of *P. cinnamomi* provides the pathogen with a diversified ability to survive and establish infection successfully. The mycelial growth within host tissue and transmit to neighbouring susceptible plants by root-to-root contact. They can subsequently produce dormant spores and zoospores. Dormant spores such as oospores and chlamydospores are resistant to harsh environmental conditions and zoospores are motile and can swim through watery soils. The motile state allows it to travel towards plants, responding to tactic gradients and attach to host tissue to initiate infection. Despite this general framework, the biochemical responses involved in the pathogenicity and vegetative development of *P. cinnamomi* is not well understood. To decipher the pathogenicity of *P. cinnamomi,* proteomics was used to take a biochemical snapshot of the mycelia, zoospores and secreted proteins from the mycelia. By doing so, a detailed profile of the pathogen was developed, providing insight into the virulence of this environmentally and economically destructive organism.

Methodology

Mycelial, zoospore and secretome sub-proteomes were purified and subjected to mass spectrometry analysis in a label-free quantification approach (independent sample preparation and data from chapter 3). The mass spectra were matched to the version 2 draft genome (see chapter 3) of *Phytophthora cinnamomi* MU94-48. The identified proteins were analysed for functional assignment using the Gene Ontology, KEGG orthologues and Interpro annotations. Gene ontology enrichment of the sub-proteomes was performed using Fisher's exact test. To determine whether the secretome contained necrosis-inducing molecules, a host (*Lupinus angustifolius)* was inoculated with the concentrated secretome. Effector P, PFAM and PHI-BASE were

used to further characterise the protein content of the secretome and identify virulence molecules such as effectors.

Result and discussion

A total of 4635 proteins were identified from the three sub-proteomes, which account for 17.7% of the predicted proteome. 1070, 698 and 278 were unique to the mycelia, zoospores and secretome respectively. The mycelia indicated a focus of maintaining saprotrophy as the enriched gene ontologies were primarily involved in metabolism and growth. The motile zoospores were enriched with proteins of energy generation, signalling, motility and response to the external environment. The zoospores require high energy production to maintain their motility and switch efficiently between motile and vegetative states. Signalling molecules and responses to nitrosative stress were enriched indicating continual response to a fast-changing environment. Additionally, nitric oxide response is required to cope with nitric oxide accumulation in the early stages of infection. The mechanistic biochemistry of zoospore motility was also mapped, showing enrichment of motor complex components. The secretome contained a repertoire of pathogenesis-related molecules including esterases, cell wall degrading enzymes and hydrolases. The secretome elicited a necrotic response in the *L. angustifolius* roots, confirming the presence of virulence-associated molecules possibly novel effectors. Upon further characterisation, homologs to known elicitins, protease inhibitors and adhesive molecules were identified.

Implication

Understanding the biochemistry of phytopathogenic organisms such as *P. cinnamomi* is imperative to the subsequent development of sustainable management and control plans. With the emergence of chemical resistance, defining important pathogenicity factors including virulence molecules and effectors will provide precedence for more targeted treatment options and overcome such issues. The hypersensitive response observed on the *L. angustifolius* root tissue provides an avenue for purification and characterisation of these effector-like proteins that can be subsequently used for plant protection purposes such as resistance gene breeding.

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Comparative sub-cellular proteome analyses reveals metabolic differentiation and production of effector-like molecules in the dieback phytopathogen *Phytophthora cinnamomi.*

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Abstract

Phytopathogenic oomycetes pose a significant threat to global biodiversity and food security. The proteomes of these oomycetes likely contain important factors that contribute to their pathogenic success, making their discovery crucial for elucidating pathogenicity. *Phytophthora cinnamomi* is a root pathogen that causes dieback in a wide variety of crops and native vegetation world-wide. Virulence proteins produced by *P. cinnamomi* are not well defined and a large-scale approach to understand the biochemistry of this pathogen has not been documented. Soluble mycelial, zoospore and secreted proteomes were obtained and label-free quantitative proteomics was used to compare the composition of the three sub-proteomes. A total of 4635 proteins were identified, validating 17.7% of the predicted gene set. The mycelia were abundant in transporters for nutrient acquisition, metabolism and cellular proliferation. The zoospores had less metabolic related ontologies but were abundant in energy generating, motility and signalling associated proteins. Virulence-associated proteins were identified in the secretome such as candidate effector and effector-like proteins, which interfere with the host immune system. These include hydrolases, cell wall degrading enzymes, putative necrosis-inducing proteins and elicitins. The secretome elicited a hypersensitive response on the roots of a model host and thus suggests evidence of effector activity.

Key words

Oomycete, *Phytophthora cinnamomi,* dieback, proteome, zoospore, secretome, virulence, effectors

Introduction

Phytophthora cinnamomi is a soil-borne phytopathogenic oomycete that causes significant economic and environmental losses world-wide. In Australia, the pathogen causes dieback on native vegetation species as well as major horticultural crops such as avocados, macadamia, pineapple and stone fruits [1]. There is no evidence of genetic resistance being used to combat dieback disease, hence it is largely controlled by the use of the oomyceticide phosphite which can only slow down the progression of disease severity and spread [2]. The mode of action of phosphite is not well understood and the application of this chemical to crops and vegetation is highly labour intensive as it is injected manually into the trunks of susceptible trees or applied by foliar spray. These methods are implemented in susceptible native trees and several avocado varieties globally [2,3]. Evidence of emerging resistance to phosphite has been shown in *P. cinnamomi* isolates obtained from avocado orchards [4]. Several *P. cinnamomi* isolates have been screened for phosphite sensitivity *in vitro* and *in planta,* with varying sensitivities indicated by growth inhibition and colonizing ability [4,5]. There is a need to better understand the pathogenicity of this system to develop more sustainable methods of combatting dieback disease.The lifecycle of *P. cinnamomi* is characterised by several forms including vegetative and reproductive stages [6]. Briefly, vegetative mycelia grow within host tissue where they mature into sexual fruiting bodies called sporangia which in turn produce motile sexual cells called zoospores.

Zoospores travel through watery soils with the aid of flagellum until they encounter the roots of a susceptible host [7]. Once contact is achieved, the zoospores begin to secrete an arsenal of proteins to initiate the formation of dedicated structures. A protective cyst forms on the surface of the root tip from which sprout appressoria that invade the plants tissue, transporting effectors and nutrients between host and pathogen.

Transcriptomics is the method of choice for comparative gene expression analysis in many studies [8].Transcriptome analysis are relatively quantitative and can be cell-specific but do not directly inform on their protein products, localisation and protein abundance may differ from their transcripts. The proteome can be used to qualitatively and quantitatively determine protein production informing on real-time biological functions in different life stages, tissues and cellular compartments of the pathogen [9]. Traditionally, genomic approaches such as screening and prediction have been used to identify virulence factors in phytopathogens. However, proteomics can also be used to capture virulence factors in oomycetes and assist in both the discovery and validation of potential candidates [10,11]. Virulence factors such as effectors are proteins that are released by the pathogen and assist in infection by damaging host tissue or interfering with host immunity. In oomycetes, effectors include necrosis inducing proteins, RXLR and CRINKLER effectors and elicitins [12].

The virulence factors that contribute to the pathogenic success of *P. cinnamomi* have not been previously studied. The present study used mass spectrometry-based proteomics to elucidate the biochemistry of three sub-proteomes of *P. cinnamomi.* The mycelia, secretome and zoospore sub-proteomes were qualitatively and quantitatively analysed to gain an understanding of which key factors are abundant that contribute to the virulence of this pathogen. Furthermore, a cell-free secretome preparation demonstrated necrosis on lupin (*Lupinus angustifolia*) roots suggesting the presence of necrosis inducing factors. The secretome was further profiled to determine the composition of effector-like proteins that may function to dictate the outcome of infection. By studying each of these stages of growth separately and identifying these virulence-associated proteins, we provide biochemical snapshot of the organism and identify important factors that contribute to pathogenicity.

Experimental procedures

Phytophthora culture and preparation

Phytophthora cinnamomi (MU94-48) cultures were obtained from the Centre of Phytophthora Science and Management (Murdoch University, Australia) and maintained by passaging through *Malus domestica* cv. Granny Smith fruit [13,14]. Mycelia were grown on V8 juice (Campbells) agar with growth periods of 5-7 days in the dark at room temperature. Mycelia were scraped off the surface of the plates and placed into Erlenmeyer flasks containing 40 mL of Ribeiros Minimal Media [15]. Liquid cultures were incubated for 3 days in the dark at room temperature. The mycelia, zoospore and the extracellular proteome were extracted and purified as previously described [12,14,16–18]. Mycelia were harvested by centrifugation at 4000g for 30 minutes and washed twice with water. The liquid media was passed through a 0.22 µm filter to remove mycelial fragments and obtain the extracellular proteome. Zoospores were produced as previously described [14]. Purified mycelia, extracellular and zoospores proteins were snap-frozen in liquid nitrogen and freeze-dried. All sub-proteomes were prepared in triplicate.

Protein extraction and digestion

Dried mycelia and zoospores were ground to a fine powder using beads and Biosprint shaker (Qiagen). 300 µl of extraction buffer (25 mM Tris-HCl pH 7.5, 0.25% SDS, 50 mM Na_2PO_4, 1 mM Na_2F, 50 µM Na_3VO_4 and 1 mM PMSF in the presence of a protease inhibitor cocktail (Sigma)) was added to the total cell lysates and incubated for 30 min on ice with occasional vortexing. Samples were centrifuged at 15000 g for 30 minutes at 4 °C and the supernatant was transferred to a new tube. The dried extracellular proteins were resuspended in 3 mL of water and proteins from the three sub-proteomes

were precipitated with 6 volumes of acetone. Samples were initially digested with trypsin for 3 hours at 37 °C to assist in solubilising the pellet, reduced and alkylated with 50 mM tris (2-carboxyethyl)phosphine (Thermo Scientific, Waltham) and 200 mM methyl methanethiosulfonate (Sigma, St Louis) respectively. Samples were digested again overnight at 37°C with trypsin (Sigma, St Louis) at a ratio of 1:10, subsequently desalted on a Strata-X 33 um polymeric reverse phase column (Phenomenex, Torrance, CA, USA) and dried in a vacuum centrifuge.

Mass spectrometry

Samples were analysed by electrospray ionisation mass spectrometry using a Thermo UltiMate 3000 nanoflow UHPLC system (Thermo Scientific) coupled to a Q Exactive HF mass spectrometer (Thermo Scientific). Approximately 1 µg of peptides were loaded onto an Acclaim™ PepMap™ 100 C18 LC Column, 2 µm particle size x 150 mm (Thermo Scientific) and separated with a linear gradient over 190 minutes of water/acetonitrile/0.1% formic acid (v/v).

Qualitative, quantitative and functional analysis

Label-free quantification was performed using the Proteome Discoverer 2.3 using the label-free precursor quantification workflow template [19]. Spectra were matched against the *P. cinnamomi* MU94-48 genome [14]. Sub-proteomes were relatively quantified using the default precursor ion quantifier. For identification, proteins with one peptide identified were used and for quantification proteins with two or more peptides were used. The FDR was set at <1. The criteria used for significant differential abundance were $p < 0.05$. Sub-proteome enrichment was confirmed using WoLFPSort 0.1. Gene ontologies were assigned using Interpro Scan 86.0. Gene Ontology enrichment within and between sub-proteomes was determined using Fisher's exact test. To further examine protein function, KEGG orthologues and Interpro annotations were used. To further characterise the content of the extracellular proteome, the identified protein sequences were refined using SignalP 5.0, EffectorP 3.0, PFAM 34.0 and PHI-Base 4.12 for prediction of secreted proteins (the secretome) including candidate effectors and virulence proteins.

Necrosis induction assay

To determine whether the secretome is able to induce a necrotic response on a host, *Lupinus angustifolia* (Tanjil) was inoculated with the purified secretome. Freeze dried secretome (prepared as above) was resuspended in 3 mL of water and left to solubilise for one hour. The protein content was measured using a nanodrop and concentration was made up to 8 mg/mL and 500 µl of the secretome was used. A control was prepared with the growth media and an additional control containing 8 mg/mL of BSA was used to ensure the concentrated protein did not cause an effect on the plant. A protease treated secretome sample and control were also included to confirm that any potential lesion caused by the secretome was a result of protein rather than other molecules such as secondary metabolites. The samples with 10 mM dithiotheitol at 60 °C for 1 hour and subsequent trypsin treatment overnight at 37°C. *L. angustifolia* seeds were germinated by soaking in water and left for three days in a plastic container lined with moist Whatmann paper at room temperature. The seedlings were inoculated with the secretome by immersing the root tips into 1.5 mL Eppendorf tubes containing the secretome. Response from the secretome was measured by scoring the severity of the lesion colour and size, three days after inoculation. Lesion scores ranged from zero (no observable lesion) to three (dark brown necrotic root tips).

Results

Protein identifications in the sub-proteomes

Mycelia, zoospore and extracellular proteins were selected for proteomic analysis as they reflect vegetative and motile stages of the pathogen. Using high confidence peptides, 4635 proteins from the *P. cinnamomi* annotation were identified (Table 1). The total protein identifications for each sub-proteomes are shown in Supplementary material 1 and 2. This accounts for 17.7% of the predicted annotated genes in the *P. cinnamomi* genome [14]. The remaining predicted proteome may be undetected because the genes are not all expressed, the proteins are present in other life stages, the proteins are not malleable for detection by mass spectrometry or may be in low abundance. Of these, 1070, 698 and 278 were unique to the mycelia, zoospores and extracellular proteome respectively (Figure 1). A high proportion of 1803 accounting for 38.9% of identified proteins was common between the mycelia and zoospores, while 2.2% and 1.2% were common between the mycelia and extracellular proteome, and extracellular proteome and zoospore respectively. 629 proteins were shared between all three sub-proteomes. Sufficient enrichment was achieved as indicated by localisation prediction using WoLFPSort (Supplementary material 3). Localisation prediction analysis of mycelia and zoospores proteome fractions revealed 5% and 6% identified proteins predicted to be secreted respectively, whereas the extracellular proteome had 30% predicted extracellular proteins. Further scrutiny of the extracellular proteome indicates that 32% of detected proteins from the extracellular proteome contained signal peptides, as opposed to the whole predicted proteome which has a total of 7.7% predicted proteins with SignalP.

Table 1. Protein identifications and spectral matches of sub-proteomes

	Total proteome	Mycelia	Zoospore	Extracellular
Total proteins identified ≥1 peptide	4635	3605	3184	1064
Proteins identified with ≥2 peptides	3413	2567	2296	646
No. spectra identified	478730	175104	178653	124973
% spectra identified	48%	59%	56%	22%
FDR	<1%	<1%	<1%	<1%

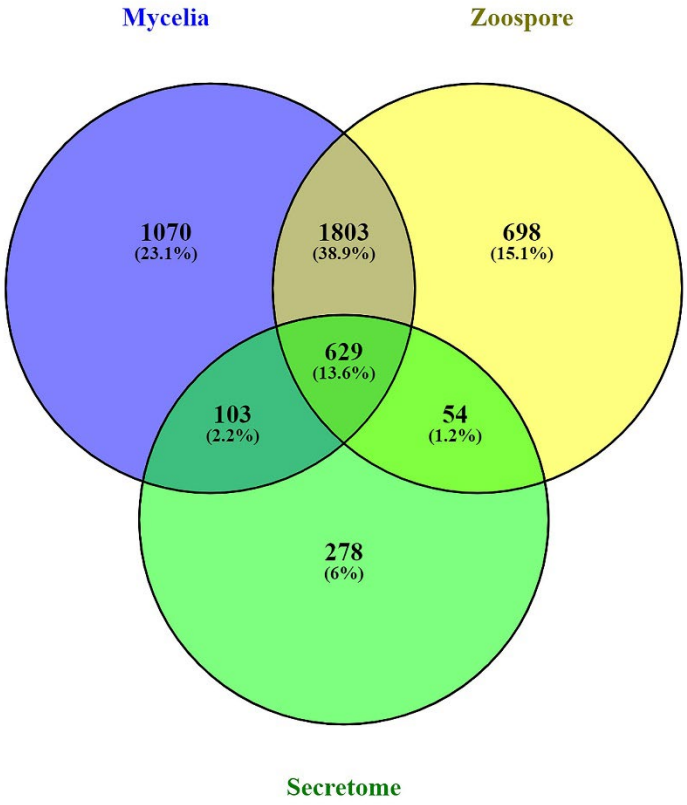

Figure 1. Sub-proteome identification of *Phytophthora cinnamomi* indicating number of unique and common proteins across the mycelia, zoospores and secretome.

The mycelia metabolism focuses on saprotrophy

The mycelia must continually obtain nutrients for saprophytic growth and successful infection of neighbouring host tissue. The functional composition of the 1070 proteins unique to the mycelia was significantly enriched with Gene Ontologies (GO) associated with metabolic processes and cellular functioning for nutrient metabolism and growth (Table 2).Transporters such as amino acid/ polyamine (IPR00229), metal ion transporters (K14686, IPR00852), inositol and sugar transporters (IPR00366), ABC transporters (K05643) and sulphate transmembrane transporters and permeases (GO:0015116, K03321) were found in the mycelia. Sugar, carbohydrate, lipid and amino acid metabolic processes were enriched in the mycelia (GO:0006000, GO:0005975, GO:0006629, GO:0044255, GO:0006520), including phospholipid, glycerol and fatty acid metabolism and biosynthesis (K13356, IPR0212, IPR01312, IPR02228), indicating metabolism of nutrients. Additionally, biosynthesis related to cell proliferation was also abundant in the mycelia (GO:0009058, GO:0007093). These include aminotransferases (K00814), proteins associated with chromosome separation such as condensing (GO:0051304) and DNA synthesis and cell division enzyme including thymidine kinase (K08866). The abundance of these proteins suggests that the primary biochemical functions of the mycelia focuses on nutrient acquisition and metabolism during vegetative growth.

Table 2. Top 10 significantly enriched GOs of the mycelia, zoospores and extracellular sub-proteomes by fisher's exact test ($p < 0.05$).

GO ID	Description	% of total GOs
Mycelia		
GO:0003824	Catalytic activity	4.56
GO:0005515	Protein binding	6.08
GO:0016887	ATP hydrolysis activity	2.74
GO:0006629	Lipid metabolic process	1.82
GO:0009058	Biosynthetic process	1.52
GO:0061630	Ubiquitin protein ligase activity	1.22
GO:0004672	Protein kinase activity	1.22
GO:0004674	Protein serine/threonine kinase activity	1.22
GO:0016579	Protein deubiquitination	1.22
GO:0006468	Protein phosphorylation	1.22
Zoospores		
GO:0005524	ATP binding	8.03
GO:0003924	GTPase activity	4.82
GO:0006468	Protein phosphorylation	2.01
GO:0016787	Hydrolase activity	2.81
GO:0016887	ATP hydrolysis activity	2.41
GO:0030286	Dynein complex	2.01
GO:0008569	Minus-end-directed microtubule motor activity	2.01
GO:0060271	Cilium assembly	1.20
GO:0006397	mRNA processing	1.20
GO:0034464	BBSome	1.20
Secretome		
GO:0004553	Hydrolase activity, hydrolyzing O-glycosyl compounds	13.95
GO:0005975	Carbohydrate metabolic process	12.79
GO:0042545	Cell wall modification	9.30
GO:0030599	Pectinesterase activity	9.30
GO:0005515	Protein binding	5.81
GO:0071704	Organic substance metabolic process	2.33
GO:0030246	Carbohydrate binding	2.33
GO:0046558	Arabinan endo-1,5-alpha-L-arabinosidase activity	2.33
GO:0031221	Arabinan metabolic process	1.16
GO:0004869	Cysteine-type endopeptidase inhibitor activity	1.16

The zoospore proteome is enriched in proteins that are associated with energy generation, motility and signalling.

The zoospores play an important role in the infection process as they enable spread of disease and can initiate the infection process by encysting on plant root tissue. The enriched GOs in the proteins unique to the zoospores were mostly associated with energy production and motility (Table 2). The full list of enriched gene ontologies for these sub-proteomes is shown in Supplementary material 2. Major energy generation and secondary signalling messengers included ATP (GO:0005524, GO:0016887) and GTPases (GO:0003924). Complexes associated with motility include dynein (GO:0030286), cilium assembly (GO:0060271, GO:0030992, GO:0042073), and the BBSome (GO:0034464). The proteins associated with these ontologies are domains of the motor complex (K10408, K16746, IPR01104, IPR02878, IPR03272) and constituents of intraflagellar transport and signalling (IPR029600). In addition, putative hydrolases (GO:0016787) were also enriched in the zoospores including glycoside hydrolases (IPR00154) and acid phosphatases (KO22390). Quantitative comparison between the zoospores and mycelia indicated an abundance in proteins associated with RNA/ protein binding and modification (Figure 2). An increase in abundance of response to nitrosative stress (GO:0051409) and nitric oxide dioxygenase activity (GO:0008941) suggests preparation to defend against host immunity

[20,21]. These proteins along with the abundance in protein phosphorylation play roles in signalling in response to external stimuli.

Figure 2. GOs that are over- and under-represented in the zoospore proteome. The X-axis indicates the log 10 ratio of the abundance ratio of zoospores to the mycelia labelled with their associated GOs. The size correlates with the number of genes annotated with each GO. A full list of significantly differentially abundant proteins and their associated GOs are shown in Supplementary material 2.

Quantitative analyses between the zoospores and mycelia also demonstrated that metabolic processes such as cellular and lipid metabolism were down-regulated in the zoospores. Metabolic processes such as lipid metabolism (GO:0006629), amino acid metabolism (GO: GO:0009072) and cellular metabolic processes (GO:0044237) were lower in abundance. RNA binding and modification (GO:0003723, GO:0009451) was over-represented in the zoospores where these proteins were involved in post-transcriptional modification, translin transport for recombination (IPR002848) and ribonucleases (IPR016068). Protein ubiquitination (GO:0016567), phosphorylation (GO:0006470) and protein binding (GO:0005515) were also higher in abundance in the zoospores with proteins specific to ubiquitin ligase components such as HECT domains (IPR035983) and SPRY domains (IPR003877) which have important roles in protein activation and signalling. This indicates that zoospores are geared towards energy production to fuel motor activity and assembly of structural components along with rapid response to external stimuli.

Analysis of the extracellular proteome and refinement of the secretome proteome reveals putative effectors and enrichment in CWDEs.

In other *Phytophthora* species, the extracellular proteome contains a plethora of virulence-associated molecules that interfere with host immunity and assist in successful infection. The mycelia produce this set of proteins to continually degrade host tissue, dampen host immunity and facilitate the spread of disease to neighbouring roots. The extracellular proteome was enriched with GOs involved in cell wall degradation and necrosis (Table 2). Cell wall modification proteins relating to structural components of plants such as pectinases (GO:0030599, K01051), proteins associated with arabinan and carbohydrate metabolic processes (GO:0046558, GO:0019566, GO:0031221, GO:0005975) and hydrolases (GO:0016787, GO:0004553) facilitate host penetration by breaking down plant tissue. Pectin associated proteins included those involved in pectin lyase folding (IPR012334), catalytic activity (IPR000070), proteins acting on active sites (IPR033131) and pectin associated virulence factors (IPR011050). Hydrolases acting on glycosidic bonds (K01184, IPR000743, IPR000490, IPR017853), concanavalin lectins (K20844, IPR013320) and acid phosphatase hydrolases (K22390, IPR008963, IPR041792) were enriched in the extracellular proteome. Cysteine-type endopeptidase inhibitors (GO:0004869, IPR000010) were identified, which are characteristic of many effector proteins.

Table 3. Candidate effector prediction of the *P. cinnamomi* secretome using SignalP, EffectorP and PFAM.

	Whole genome annotation	Secretome
Total proteins identified	26151*	1064
SignalP		
Predicted signal peptide	2010	340
EffectorP		
Predicted cytoplasmic effector	**	296
Predicted apoplastic effector	**	107
Predicted cytoplasmic/ apoplastic effector	**	19
Predicted non-effector	**	642
PFAM		
Cysteine-rich secretory proteins (CAP)	47	16
Necrosis-inducing proteins	73	8
Proteases	49	0
Pectate lyase	38	6
RXLR	77	1
Crinkler	31	0
Elicitin	57	14

* Total proteome annotation

** As EffectorP is most effective at predicting effector proteins alongside enrichment or *in planta* data, it was not applied to the whole genome.

To refine the extracellular proteome into the secretome and identify potential effector molecules, the proteins detected in the extracellular proteome were subjected to the signal peptide and effector prediction softwares, SignalP and EffectorP (Table 3). 340 extracellular proteins has predicted signal peptides and 422 of the 1064 proteins were predicted as cytoplasmic and/or apoplastic effectors, which also uses signal peptides as effector criteria. The assigned PFAM domains of the proteins within the secretome demonstrated the presence of several types of effectors including 16 cysteine-rich secreted proteins, 8 necrosis inducing proteins, 14 elicitins and one protein with an RXLR motif (Table 3). When searched against the PHI-Base database, several homologs to effectors in other *Phytophthora* species were found, including elicitins INF1 and INF2A, protease inhibitors such as EPI1 and EPI2, suppressors of elicitor mediated plant defence response such as GIP and several adhesive and necrosis inducing

proteins such as PsoNIP, CBEL-GP34, Pc129485 and PcPL. This suggests that the secretome is enriched with molecules with putative functions in host penetration and immune system manipulation.

Table 4. Homology of *P. cinnamomi* secreted proteins to known *Phytophthora* effectors using PHI-Base. A full list of PHI-Base hits to the *P. cinnamomi* secretome is shown in Supplementary material 4.

P. cinnamomi gene ID	Effector ID	Description
	P. infestans	
34115|estExt_Genemark1.C_2210010	INF1	Elicitin that induces a hypersensitive response in potato
73042|fgenesh1_pg.221_#_16		
93043|fgenesh1_kg.43_#_38_#_Locus12203v1rpkm4.23		
93203|fgenesh1_kg.47_#_7_#_Locus4515v1rpkm40.01		
96866|fgenesh1_kg.141_#_16_#_Locus10244v1rpkm8.27	INF2A	
116215|estExt_fgenesh1_pg.C_590073	EPI1	Protease inhibitor of P69B subtilisin-like serine protease in tomato
148089|gw1.32.186.1		
313058|estExt_Genewise1Plus.C_590249		
107067|estExt_fgenesh1_pm.C_970022	EPI10	
307297|estExt_Genewise1Plus.C_260341		
314066|estExt_Genewise1Plus.C_650107		
99941|fgenesh1_kg.857_#_3_#_Locus10372v1rpkm7.98		
98086|fgenesh1_kg.200_#_18_#_Locus19844v2rpkm0.34		
313378|estExt_Genewise1Plus.C_610207	EPI2B	Protease inhibitor targeting cysteine proteases C14, PIP1 and RCR3 in tomato
95628|fgenesh1_kg.98_#_25_#_Locus11013v1rpkm6.49		
	P. sojae	
217079|e_gw1.19.367.1	GIP1	Suppresses elicitor mediated defence response in plants
260346|e_gw1.593.10.1		
246955|e_gw1.170.26.1	GIP2	
232501|e_gw1.73.90.1		
232547|e_gw1.73.128.1		
246955|e_gw1.170.26.1		
322876|estExt_Genewise1Plus.C_1520005		
574163|MIX23955_15_97		
585554|MIX35346_4_39		
92737|fgenesh1_kg.38_#_59_#_Locus12084v1rpkm4.40		
99743|fgenesh1_kg.475_#_1_#_Locus15159v1rpkm1.68		
113206|estExt_fgenesh1_pg.C_130064	PsXEG1	Apoplastic endoglucanase
232304|e_gw1.72.180.1		
253845|e_gw1.255.36.1		
68102|fgenesh1_pg.72_#_72		
90808|fgenesh1_kg.13_#_53_#_Locus11690v1rpkm5.07		
482294|CE151240_54	PsojNIP	Necrosis inducing protein
	P. parasitica	
207345|e_gw1.2.728.1	CBEL-GP34	Cell wall glycoproteins that induce necrosis and defence responses in host
207378|e_gw1.2.738.1		
76042|fgenesh1_pm.2_#_264 89138|fgenesh1_kg.2_#_206_#_Locus5976v1rpkm27.05		
89900|fgenesh1_kg.6_#_83_#_Locus15420v1rpkm1.57		
91921|fgenesh1_kg.25_#_27_#_Locus7572v1rpkm17.82		
91922|fgenesh1_kg.25_#_28_#_Locus11823v1rpkm4.82		
96245|fgenesh1_kg.120_#_11_#_Locus1031v2rpkm3.31_PRE		
98650|fgenesh1_kg.239_#_12_#_Locus11880v1rpkm4.74		
	P. capsici	
285566|estExt_Genewise1.C_1150039	Pc129485	Nep1-like protein
319952|estExt_Genewise1Plus.C_1150037		
19441|gm1.19441_g	Pc107869	
211869|e_gw1.8.695.1	PcPL1	Pectate lyase
89577|fgenesh1_kg.4_#_162_#_Locus17166v1rpkm1.04		
125848|gw1.44.6.1	PcPL15	
241043|e_gw1.121.69.1		
90231|fgenesh1_kg.8_#_124_#_Locus6106v2rpkm0.00_PRE		
94152|fgenesh1_kg.64_#_9_#_Locus13537v1rpkm2.71	PcPL20	

To determine if the secretome contained candidate effectors that induce a hypersensitive response in plants, Lupin seedlings were inoculated with a cell free secretome preparation. *P. cinnamomi* MU94-48 infects and causes dark necrotic lesions on *Lupinus angustifolius* roots [22]. Exposure of the seedlings to the cell free secretome caused a hypersensitive response where dark lesions were developed three days post-inoculation (Figure 3). The secretome was also treated with a protease to confirm that the HR was caused by proteins only, however the protease treated control caused a lesion response in the root.

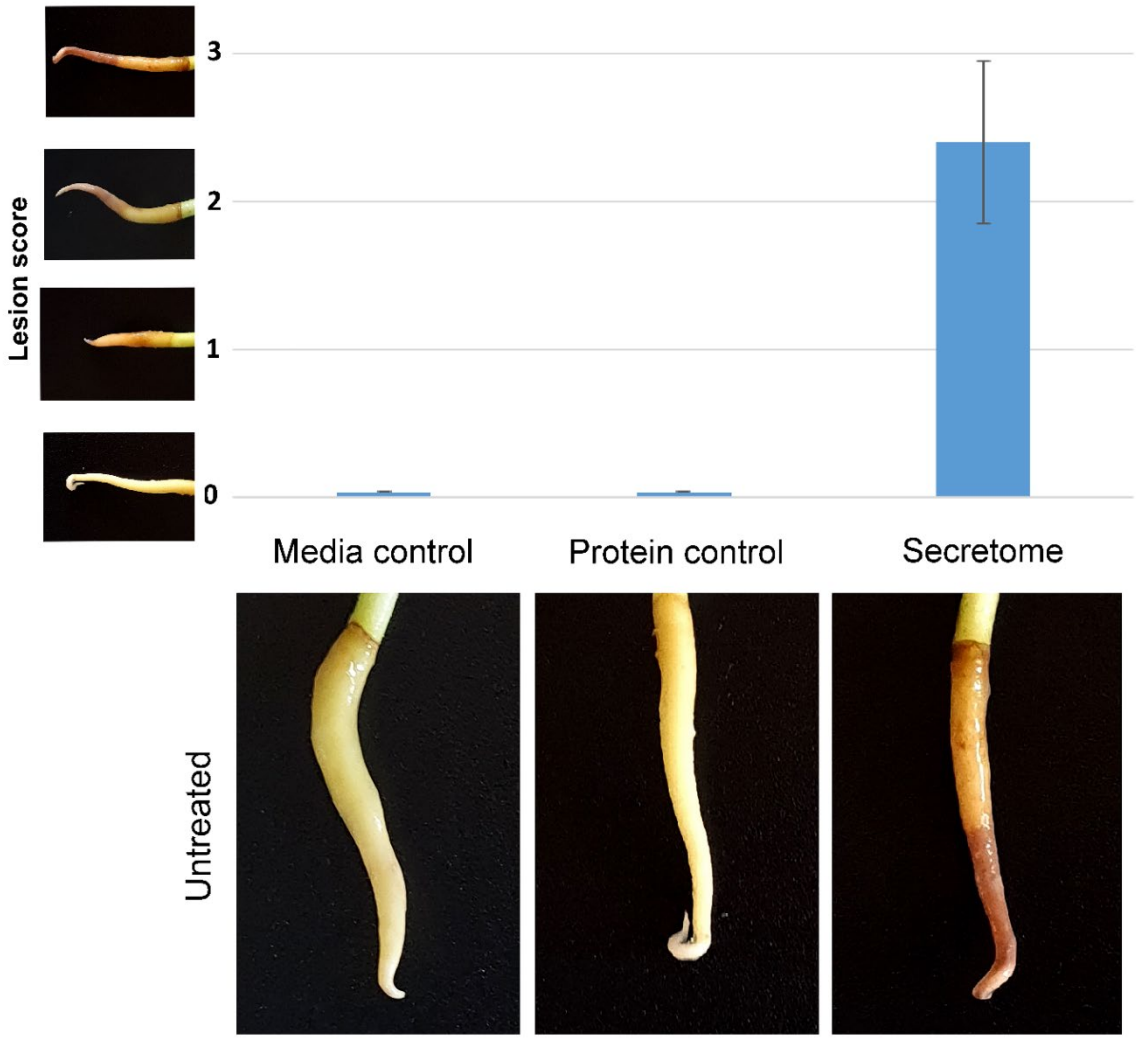

Figure 3. Lesion score and necrosis on *L. angustifolia* roots caused by the cell-free secretome extract of *P. cinnamomi.* A media and a equivalent concentration protein control were used. Lesions were scored from zero to three (as indicated by lesion images on y-axis) by the colour and size of necrotic tissue.

Discussion

Phytophthora cinnamomi has caused significant losses in crops such as avocado, macadamia and pineapples, in addition to destroying huge areas of natural vegetation and ecosystems world-wide [6]. Not much is known about the molecular mechanisms *P. cinnamomi* infection. Here, we profiled the sub-proteomes of mycelia, zoospores and the secretome using label-free quantitative proteomics to obtain a biochemical snapshot of *P. cinnamomi* that could be used to understand pathogenic success. Although the sub-proteomes were obtained from *in vitro* growth, we present a model of key biochemical processes inferred from protein abundance in zoospore, mycelial and secreted proteins in relation to host tissue (Figure 4).

Mycelia not only grow within root tissue and obtain nutrients through the roots of susceptible plants but are also transmitted to neighbouring plants by direct root-to-root contact [1]. Several types of transporters are used by oomycetes to acquire nutrients from the extracellular space and host tissue [23]. Fifty two transporter proteins were identified in the mycelia, potentially showing a priority of protein mobilisation. This is similar to the mycelial proteomes of *P. pisi*, *P. sojae* and *P. ramorum,* where proteins associated with metabolism, biosynthesis and nutrient transport were enriched or higher in abundance [24,25]. The enrichment of metabolic processes and cellular reproduction associated proteins in the mycelia indicate that once nutrients have been taken up by the pathogen and either stored, respired without processing or they are processed and utilised to facilitate growth (Figure 4).

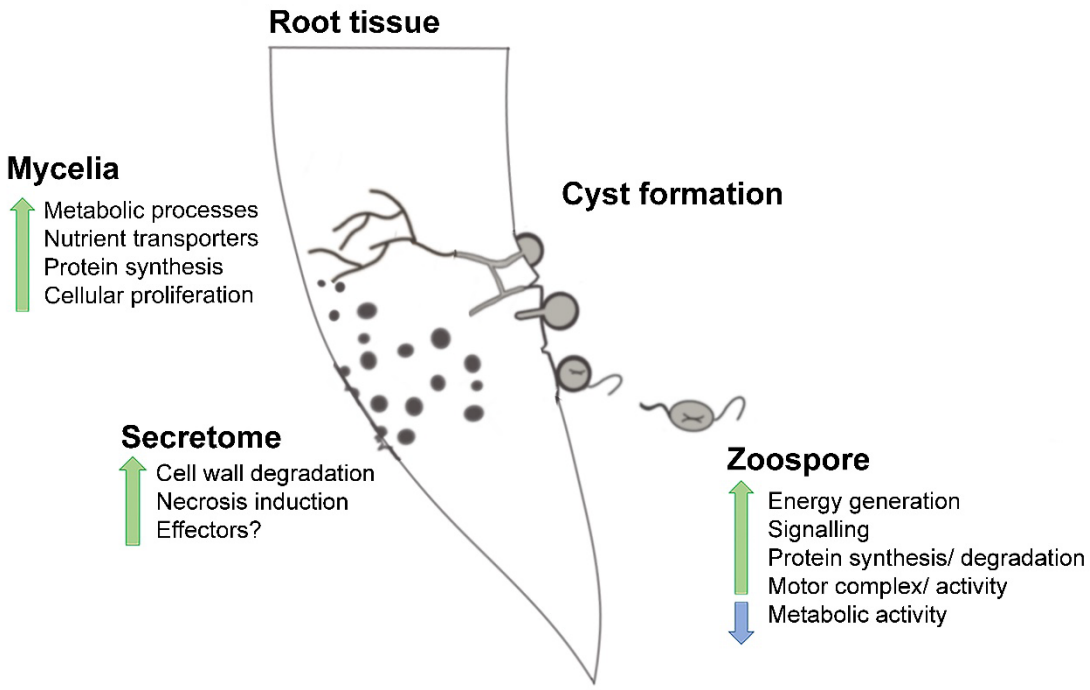

Figure 4. A proposed model of biochemical functions occurring at different sub-proteomes and developmental stages of *P. cinnamomi*. Arrows indicate relative enrichment in each sub-proteome.

When conditions favour growth, the mycelia form sporangia that produce zoospores [6]. Zoospores have an anterior whiplash and posterior tinsel flagella, which enable them to swim through in moist soil towards potential hosts by tactic gradients [26,27]. The zoospores are important in the process of infection as they are the first points of contact with the host. The enriched GOs in the zoospores included many associated with energy production such as ATP binding, protein phosphorylation and ATP hydrolysis. Here we hypothesise that there are several needs for zoospores to produce copious amounts of energy. Firstly, zoospore motility requires constant energy production to maintain. Their posterior flagella propels the cells forward and the anterior flagellum is used to steer until they have the opportunity to encyst on the surface of host tissue [28]. Secondly, once zoospores infect the host, they can switch back to vegetative mycelia where they must derive energy from glycolysis and drive processes that enable the survival of the pathogen within the plant tissue [25]. The reduced abundance of proteins associated with cellular and lipid metabolism reflects the utilisation of energy generation towards differentiation and motility rather than growth which does not increase until germination of mycelia within host tissue (Figure 4).

The zoospores also showed an enrichment in RNA binding and modification, chromosomal recombination, protein serine-threonine phosphatase activity, dephosphorylation, protein binding and ubiquitination. This could indicate simultaneous high protein synthesis activity and degradation and

may be a response to fast-changing environmental conditions by co-regulating protein synthesis and protein breakdown for proteome adaptation [29,30]. Enrichment of proteins associated with response to nitrosative stress can be a result of three factors. Nitric oxide (NO) has been shown in pathogenic fungi and oomycetes such as *Phycomyces blakesleeanus* to act as a light sensor molecule during light-mediated sporulation [20]. Sporulation of *P. cinnamomi* zoospores *in vitro* is light dependent which may use NO as a signalling molecule during this process [20]. This may also be facilitated by the enrichment in signalling molecules such as phosphorylation which are abundant in the zoospores. NO is also known to be produced by phytopathogens to aid in early infection such as the oomycete *Bremia lactucae,* where it is important for penetration of the host cell surface [31]. The nitrosative stress response and nitric oxide dioxygenase activity are important here as even though NO production favours the pathogen during the initial infection, NO accumulation can affect fungal and oomycete growth [32]. Lastly, an enrichment in response to nitrosative stress is also utilised in the context of plant production of NO, which is produced by the host as a toxifying defence mechanism [33].

Zoospores were also enriched with motor-related proteins such as minus-end-directed microtubule motor activity, cilium assembly, BBSome, dynein complex and kinesin binding proteins. The microtubule is driven by ATP hydrolysis, which was also found to be highly abundant in the zoospores. In oomycetes, dynein complexes are force generated motor proteins that play a crucial role in the movement of the microtubule that drives motility of cilia and flagella [34]. The BBsome component (analogous to the Baedet-Biedl Syndrome proteins involved in cilia development) in this complex is important in mediating cilia homeostasis in response to stimuli [35]. The flagella are controlled by motor proteins such as kinesin, which drive the motions of the two types of flagella.

GOs that correspond to proteolysis, cell morphogenesis, tubulin and proteins associated with endocytic recycling were over-represented in the zoospore proteome. When zoospores come into contact with a host, they adhere to the cell surface and form cyst structures [6]. Proteolytic enzymes can be used in two ways during this process. They are required during cell morphogenesis as the zoospores encyst. Host cell walls are protein-rich therefore proteases are used by the pathogen to degrade cell walls which are subsequently utilised by the mycelia as a nutrient source for growth [36]. Structural components such as tubulin are also formed as components of the appressorium that penetrates host tissue and allow the pathogen to grow within the plant [25,37]. This data supports our proposed model that zoospores generate energy to fuel their motility, whilst attempting to dampen the responses of a potential host.

Extracellular proteins and the secretome are of a significant interest due to the mobilisation of effectors into host tissue. Effectors have been identified in many *Phytophthora* species, where they play important roles in infection [38]. The expression of α- and β-cinnamomin elicitins have been found in various cell types of *P. cinnamomi,* of which β-cinnamomin has been shown to play some role in virulence, however the infection process is a result of a number of effector proteins [39–42]. The expression of several predicted RXLR effectors has also been demonstrated in *P. cinnamomi* when inoculated onto avocado roots [43]. The full extent of effectors of *P. cinnamomi* have not been fully characterised nor identified at the protein level.

Contamination of the extracellular proteome from cytoplasmic proteins can occur as a result of cell death and lysis [44]. Protein localisation prediction demonstrated enrichment of the extracellular proteins compared to the mycelia, zoospores and total predicted gene set of *P. cinnamomi.* Signal peptide analysis also indicated enrichment of secreted proteins in the extracellular proteome compared to the whole genome annotation. Additionally, glyceraldehyde-3-phosphate dehydrogenase, a cytoplasmic marker protein not classically found in secretory pathways was not detected in the extracellular proteome [14,45]. Although this indicates some level of contamination, this is comparable to observations seen in oomycete and fungal pathogens where cytosolic contamination is unavoidable during sample preparation [14,17,46,47].

The secretome profile of *P. cinnamomi* in this study included proteins that contribute to pathogen virulence. This includes enrichment of proteins associated with cell wall modification, carbohydrate metabolism, pectinesterase activity and carbohydrate-binding. These proteins bind and catabolise components of the cell wall that facilitate degradation of host tissue to allow the appressoria to infiltrate and begin infecting the host [48]. Pectinesterases are proteases that aid in this process by catalysing de-esterification of pectin, a major constituent of plant cell walls [49,50]. The identified pectinesterases catalyse the breakdown of pectin into pectate and methanol in plant cell walls by binding the active site and lyse the folding structure of the pectin backbone [51]. The mycelia utilise CWDE such as glycosidic hydrolases, lectin and acid phosphatase hydrolases as a means of saprophytic in the extracellular space [52]. Similarly to the secretomes of *P. pisi, P. sojae* and *P. plurivora* several other hydrolases were enriched in the secretome, which can aid host cell disruption and degradation of host defences [24,49]. In plants, cysteine-type endopeptidases are released in response to pathogen therefore we hypothesised that hydrolases expressed in *Phytophthora* function to inhibit these proteases from the host [53–55].

Searching the set of secreted proteins against the PHI-Base database indicated homology with several *Phytophthora* species (Table 4). Elicitin effectors such as INF1 and INF2A in *P. infestans* have both been shown to cause a hypersensitive response in hosts such as potato [56,57]. Several protease inhibitors such as EPI1, EPI10 and EPIC2B in *P. infestans,* which block the activity of the proteases, were homologous to proteins detected in the secretome. P69B subtilisin-like serine proteases and EPIC2B are protease inhibitors of cysteine proteases, which are expressed in tomatoes as a defence mechanism against *P. cinnamomi* [58–60]. Other effectors such as GIP1 and GIP2 in *P. sojae* suppress elicitor mediated defence responses in their hosts [61]. Homologs to PsXEG1 and CBEL-GP34 were also found, which are adhesive molecules that act on components of the cell walls of hosts in *P. sojae* and *P. parasitica* [62,63]. Homology of many virulence and effector transcripts between *P. cinnamomi* and *P. infestans* has been demonstrated, including adhesion proteins, hydrolases, proteases and elicitins [64]. Secretomes identified across many *Phytophthora species* contain a plethora of virulence proteins that contribute in some way to infection of host tissue.

Lupin seedlings were used to test whether the secretome can cause a hypersensitive response *in planta* (Figure 3). Dark lesions on the roots of the seedlings indicate that the secretome contains effector molecules. To gain a deeper understanding of the repertoire of virulence molecules in the secretome, effectorP, PFAM and PHI-Base were used to predict the types of effectors making up the secretome, and look for homology of known effectors (Table 3 and Table 4). Forty percent of the proteins identified in the secretome had predicted cytoplasmic and/or apoplastic effectors. Cysteine-rich proteins were identified in the secretome, which are characteristic of many effectors across oomycete and fungal plant pathogens [65,66]. Several putative necrosis inducing proteins were also identified along with several elicitins. One of these candidates possess an RXLR domain (o81992|fgenesh1_pm.63_#_55). The RXLR domain is found in some oomycete effector proteins which assists in translocation into host [67].

Concluding remarks

This study provides an in-depth analysis of the protein composition in the mycelia, zoospores and secretome of *P. cinnamomi*. The mycelia were found to be highly metabolically active as a means of saprophytic growth. The biochemical processes in the zoospores are geared towards their motility with an abundance of energy generation to fuel their motility. Here we mapped out the constituents of the motor complex and signalling processes of the zoospores to how they would fit in an infection model. An in-depth dissection of the zoospores has not been previously documented in any *Phytophthora* species. We also provide the first secretome profile of *P. cinnamomi* which includes virulence associated proteins and candidate effectors such as necrosis inducers and elicitins. The discovery of candidate effectors paves the way for future studies and the development of new control measures. This dataset provides a snapshot of the key factors that contribute towards the successful infection of *P. cinnamomi*

on its hosts. In future studies, this model can be investigated *in planta* to confirm the biological processes involved in infection and further understand how successful infection is achieved. Due to the emergence of phosphite resistance, there is a need for novel methods of disease management which may be achieved through the discovery of candidate virulence factors such as effectors to aid in the identification of genetic resistance in plants.

Availability of data and materials

Spectral data used for this study are available at Figshare (DOI: 10.6084/m9.figshare.19161368).

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Abbreviations

MS, Mass spectrometry; LC, Liquid chromatography, GO, Gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; CWDE, cell wall degrading enzymes; BBSome, Baedet-Biedl Syndrome proteins; NO, nitric oxide.

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Chapter 5 The oomyceticide phosphite exhibits multi-modal action in an oomycete pathosystem.

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Background

Phytopathogenic oomycetes cause the destruction of a variety of plants in natural ecosystems and several horticultural crops in agriculture including avocado, pineapple, macadamia, and stone fruits. They contribute significantly to environmental and economic losses on a global scale. Currently, the only implemented chemical used to manage dieback disease is phosphite. Phosphite can reduce the spread and severity of disease caused by *P. cinnamomi* but it cannot completely eradicate an infestation. The mode of action of phosphite is not well understood but it has been suggested that it acts both directly on the pathogen and through the host plant's immune system. In recent years, phosphite-resistant isolates of *P. cinnamomi* have emerged in avocado orchards and now pose a more significant threat to susceptible hosts. In the present study, a proteomic approach was used to gain a better understanding of how phosphite inhibits *P. cinnamomi* growth and determine how it interacts with host immunity.

Methodology

A phosphite-sensitive (MU94-48) and a resistant (CPSM366) isolate of *P. cinnamomi* were grown on phosphite supplemented media. Mycelial proteins were purified and subjected to mass spectrometry analysis for label-free quantification. Enrichment of gene ontologies and differential abundance of proteins in the sensitive and resistant isolates were used to examine the direct effect of phosphite and determine the biochemical differences between the two isolates when subjected to phosphite.

To determine the indirect mode of action of phosphite *in planta, Lupinus angustifolius* (narrow-leaf lupin) seedlings were treated with phosphite. Root tips from the untreated and treated lupin seedlings were excised, proteins extracted, and compared by label-free quantitative proteomics. Gene ontology enrichment and differential

abundance of proteins were used to inform on the metabolic differentiation between the treated and untreated seedlings.

Result and discussion

In the sensitive isolate, an enrichment of stress-related proteins was observed which was not seen in the resistant isolate. The biochemistry of the sensitive isolate showed enrichment in signalling, transporters, protein activation, and unregulated gene expression indicating that phosphite may interact with several biochemical pathways. Quantitative analysis showed a reduction in mitochondrial respiration, which is a common target for many fungicides.

The phosphite-treated lupin had significantly enriched and increased abundance in photosynthesis, photorespiration and carbon fixation. This was also reflected in the increase of gene expression and starch metabolism, and reduced glyconeogenesis. This suggests that phosphite drives the metabolic plant process. Enrichment of defence and stress-related molecules such as proteasomes, peroxidases and oxidant detoxification proteins along with several secondary metabolites such as isochorismatase, superoxide dismutase and annexins. Rather than phosphite directly priming the immune system of plants, this data suggests that phosphite acts as a substrate and drives plant metabolism and/ or defence pathways. Alternatively, the abundance of defence-related molecules could be a byproduct of a heightened metabolism.

Implication

This data presents a comprehensive snapshot of the possible mode of action of phosphite directly on the pathogen *P. cinnamomi* and on plant systems. I present a model of the induction of an oomycete system that is unable to compensate for the biochemical disruption caused by phosphite. In a plant system, phosphite causes increased metabolic activity. This poses an explanation as to how phosphite acts as a biostimulant in plant systems. As there is no alternative to phosphite and resistance is becoming more common, there is pressure to understand the mode of action of phosphite and the mechanisms in which *P. cinnamomi* has obtained resistance. The

unresolved dilemma of the mode of action of phosphite is whether the effect *in planta* on the pathogen is a result of the heightened immune system of the plant or from the pathogen up taking phosphite directly from the plant tissue. This poses an interesting question for future research.

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Proteomic analysis revealed that the oomyceticide phosphite exhibits multi-modal action in an oomycete pathosystem.

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ABSTRACT

BACKGROUND: Phytopathogenic oomycetes constitute some of the most devastating plant pathogens that cause significant crop and horticultural loss. *Phytophthora cinnamomi* is a phytopathogenic oomycete that causes dieback disease in native vegetation and a variety of crops. This pathogen can survive through harsh environmental conditions which gives it an advantage over its susceptible hosts. The only implemented chemical used to control *P. cinnamomi* is the oomyceticide phosphite. Despite its widespread use, the mode of action of phosphite is not well understood and it is unclear whether it works directly on the pathogen or through the host. Additionally, resistance to phosphite is emerging in *P. cinnamomi* isolates and other oomycete phytopathogens.

RESULTS: The mode of action of phosphite on the pathogen and through a model host was investigated using label-free quantitative proteomics. *In vitro* treatment of *P. cinnamomi* with phosphite hinders growth by interfering with metabolism, signalling and gene expression as a stress response to the oomyceticide, traits that are not observed in the tolerant isolate. When the model host *L. angustifolius* was treated with phosphite, enrichment of proteins that are associated with photosynthesis, carbon fixation and lipid metabolism in the host was observed. An increase in the production of a range of defence-related proteins was observed.

CONCLUSION: We hypothesise direct and indirect models of the multi-modal action of phosphite that directly targets the pathogen as well as alters plant metabolism and immune response.

Keywords

Phytophthora cinnamomi, oomycete, phosphite, resistance, mode of action, oomyceticide

1 INTRODUCTION

Phytopathogenic oomycetes are significant plant pathogens in natural ecosystems and agriculture. These pathogens cause substantial environmental and economic losses from plant death and management costs. *Phytophthora cinnamomi* is an oomycete that causes dieback disease in native vegetation and several crops including avocado, macadamia, pineapple, and a variety of stone fruits. This necrotic pathogen attacks the roots of susceptible hosts and causes plant death via saprophytic growth [1]. The dynamic lifecycle of this organism drives its resilience and success as a plant pathogen [2]. Similarly to other *Phytophthora spp,* its spores enable it to survive through harsh environmental conditions and thrive once conditions become more favourable. This gives the pathogen an advantage over its susceptible hosts. Characteristically, oomycetes produce an assortment of virulence molecules throughout their life cycles which facilitate the infection process [3,4].

Phosphite is the only commercially available chemical used to control *P. cinnamomi* [5]. *Inorganic phosphites* are reduced forms of phosphate that are commonly used as oomyceticides to manage diseases caused by the *Phytophthora spp.* This includes *P. cinnamomi, P. nicotianae, P. palmivora, P. capsici* and *P. infestans,* along with other oomycetes such as downy mildews such as *Pseudoperonospora humuli* and *Bremia lactucae* [6–10]. It is applied on native trees and horticultural crops by foliar spray or direct injection into the trunk where it circulates throughout the plant system [11]. In this manner, intact phosphite can be detected in plant tissue including the roots using analytical chemistry assays such as liquid chromatography mass spectrometry [12]. Phosphite cannot eradicate *P. cinnamomi* in the field but it can be used to reduce the severity and spread of the disease. It is also used as a preventative measure in uninfected areas [11,13,14]. The only other strategies implemented to combat dieback disease are the use of tolerant root stocks if they are available (such as commercially available avocado root stocks) and hygiene measures applied to vehicles and personnel, which aim to minimise spread [15–18]. *In vitro* fungicide screening of *P. cinnamomi* has shown growth inhibitory effects of chemical alternatives such as metalaxyl, fosetyl-A1, benzethonium chloride and copper salts however, these remain to be tested in the field [19,20]. As such, phosphite remains the only option for the chemical control of *P. cinnamomi.*

Despite its widespread use in dieback management, the mode of action of phosphite is not well understood. It is suggested that it acts both directly on the pathogen and indirectly through the plant host by priming the plant immune system [7,8,21–23]. It has been reported that mycelial growth and

sporulation are directly inhibited by phosphite *in vitro* [21,22]. *In planta*, *t*he increase in transcription of defence-related genes associated with the salicylic and jasmonic acid pathways in *Arabidopsis thaliana* and potato crops have been demonstrated post-treatment with phosphite [7,8,23]. The effect of phosphite on potato leaves indicates an increase in defence responses and altered metabolism, glycolysis, and carbon fixation [23]. These findings have led to the hypothesis that phosphite primes the immune system of plants for potential infection. Despite these observations, the mode of action of phosphite on the pathogen has not been defined, nor have the mechanisms of phosphite primed plant defence.

Tolerance of *P. cinnamomi* to phosphite is widespread with most reports originating from horticultural plantations and variability in phosphite sensitivity has been demonstrated *in vitro* and *in planta* [21,24]. Tolerance to phosphite has also been reported in *P. nicotianae, P. capsici, Bremia lactucae* and *Pseudoperonospora humuli* [9,10,25–27]. This is a suspected result of prolonged use of phosphite in agriculture and poses a significant threat to natural ecosystems and the agricultural industry. These tolerant isolates are driving the pressure for improved management strategies.

Oomyceticide modes of action are typically determined using transcriptomic methods, which identify changes in transcripts in response to these chemicals. This has been achieved in several chemicals applied to oomycete species such as dimethomrph application to *P.parasitica*, where transcripts informed on changes of cell wall/ membrane and cell wall synthesis [28]. Genome wide association studies can be used to identify specific genes that may infer resistance of an oomycete pathogen to a chemical. This method has been used in mefenoxam and metalaxyl resistant *P. capsici* to identify candidate genes that contribute to changes in sensitivity/resistance [29,30].

Proteomics in this context can also be used to observe the functional changes in the pathogen or host plant that are caused by oomyceticide treatment along with chemical resistance [31,32]. To elucidate the mode of action of phosphite on the pathogen and the host, we used a label-free quantitative proteomic approach to conduct a study encompassing the effects of phosphite on a sensitive and a tolerant isolate of *P. cinnamomi*, as well as investigating the effects of phosphite on the physiology in a model plant system that is susceptible to dieback. *P. cinnamomi* is used as model to understand phosphite tolerance as it is treated with phosphite in the field and tolerant isolates have emerged. By using a shotgun approach, we can obtain a snapshot of the biochemical processes that are altered as a result of treatment with phosphite and determine whether phosphite exhibits a direct and/or indirect mode of action.

2 MATERIALS AND METHODS

2.1 *In vitro* treatment of *P. cinnamomi* with phosphite

Stocks of two *P. cinnamomi* isolates, MU94-48 and CPSM366 were obtained from the Centre of Phytophthora Science and Management, Perth, Australia. MU94-48 was collected from susceptible *Eucalyptus marginata* in Willowdale, Western Australia with no history of phosphite use and CPSM366 was collected from a Western Australian avocado orchard with a history of extensive phosphite use and reduced efficacy of protection on dieback was observed [2433]. To determine the level of phosphite sensitivity of both isolates, they were grown on Ribeiro's media (control and amended with phosphite) at concentrations ranging from 0.0 µg mL^{-1} to 1000 µg mL^{-1} by placing a mycelial plug in the centre of each phosphite and control plate [34]. Mycelial cultures were incubated at room temperature in the dark. After 14 days of growth, the mycelial radial growth was measured to determine the growth inhibition. The EC_{50} and minimum inhibitory concentration (MIC) for both isolates were calculated as previously described [35]. For proteomics analysis, growth inhibition of 0% and 20% were used as untreated and sub-lethal doses of phosphite to cause a physiological effect and provide enough biomass for experimentation. Mycelia were harvested for protein extraction by scraping from the surface of the plate, snap-frozen in liquid nitrogen and freeze-dried. Lyophilised mycelia were ground using metal beads and a tissue mill (Retsch, Haan, Germany) at a frequency of 3 Hz/s for three minutes.

2.2 *In planta* treatment of *Lupinus angustifolius* and inoculation with *P. cinnamomi*

The narrow leaf lupin *Lupinus angustifolius* (cv. 'Tanjil') seeds were surface sterilised with 5% sodium hypochlorite, washed twice with 70% ethanol and washed three times with water. Seeds were placed in clear containers lined with moist Whatman paper (Cytiva, Massachusetts, USA) and left to germinate for 3 days at room temperature under natural light [36].

Three-day-old germinated seedlings were sprayed with a 0.5% pH 7 solution of phosphite (commercially used concentration and pH) (Sigma, St Louis, USA) using a hand-triggered spray bottle and untreated seedlings were sprayed with water [37–39]. Both the root and stem were sprayed for the purposes of preliminary experimentation and to ensure phosphite was absorbed throughout the plant. Seedlings were incubated on a light shelf at room temperature with a 12-hour photoperiod and seedlings were sprayed daily with water [36]. Lupin root tips were harvested one day post first treatment. 1.5 cm of root tips were excised and immediately snap frozen, freeze dry, and ground to a fine powder using metal beads and a tissue mill (Retsch, Haan, Germany) at a frequency of 3 Hz/s for three minutes. Three biological replicates (of which three root tips were collected) were used for each sample.

To test the effect of phosphite on the colonisation ability of *P. cinnamomi,* 24 hours post- spraying, the infected seedlings were inoculated with 5 mm mycelial discs of each isolate. Containers were placed back on the light shelf for three days and lesion scores from zero to three were recorded.

2.3 Protein extraction and digestion

300 µl of extraction buffer (25 mM Tris-HCl pH 7.5, 0.25% SDS, 50 mM Na_2PO_4, 1 mM Na_2F, 50 µM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail) was added to the ground mycelia and the samples were kept on ice for 30 minutes with regular gentle mixing. The crude extract was centrifuged at 20,000 g at 4 °C for 30 minutes, the solubilised proteins were decanted, and proteins were precipitated using six volumes of ice-cold acetone and incubated at -20 °C overnight [40].

300 µl of extraction buffer (125 mM Tris-HCl pH 7.0, 7 % SDS, 0.5 % PVP-40) was added to the whole ground plant material and the samples were kept on ice for 30 minutes with regular gentle mixing. The crude extract was centrifuged at 20,000 g at 4°C for 30 minutes. Proteins were purified by adding 800 µL of ice-cold methanol and 200 µL of ice-cold chloroform to 200 µL of the solubilised protein samples. Samples were vortexed, and 500 µL of water was added and centrifuged for 5 minutes at 15000g a 4 °C. The aqueous phase was removed and 500 µL of methanol was added. Samples were inverted and the supernatant was discarded. 1 mL of ice-cold acetone was added and the samples were incubated at -20 °C overnight [41].

The mycelial and root protein pellets were washed twice with ice-cold acetone and reconstituted in 200 µl of 0.5 M triethylammonium bicarbonate (pH 8.5) before reduction and alkylation with 20 µL of 50 mM tris(2-carboxyethyl)phosphine (Thermo Scientific, Massachusetts, USA) and 10 µL 200 mM methyl methanethiosulfonate. Samples were tryptically digested overnight at 37 °C at a ratio of 1:10, subsequently desalted on a Strata-X 33 um polymeric reverse phase column (Phenomenex, Torrance, CA, USA) and dried in a vacuum centrifuge [42].

2.4 Mass spectrometry and data analysis

1 µg of each sample was loaded on columns and peptides were resolved with a gradient of 10-40% acetonitrile (0.1% formic acid) at 300 nL/min over 90 minutes and eluted through a nanospray interface into a Q-Exactive Orbitrap mass spectrometer (Thermofisher Scientific). Qualitative and label-free quantification was performed using Proteome Discoverer 2.3 [43]. Mass spectra from the *in vitro* assay were matched to the *P. cinnamomi* MU94-48 genome consisting of 26,151 protein-coding sequences [44]. The proteomes between phosphite-treated MU94-48 and CPSM366 were compared to simultaneously gain insight into the effects of phosphite on sensitive *P. cinnamomi* and understand the differences between phosphite sensitivity and tolerance. The mass spectra obtained from the *in planta*

assay were matched to the *L. angustifolius* genome consisting of 39,339 protein-coding sequences [45]. For protein identification, 1 or more 95% confidence peptides were used. For label-free quantification, proteins with 2 or more 95% confidence peptides were used and for significant differential abundance a p-value threshold of <0.05 was used as previously described [42,46]. Ratios and p values for quantitative analysis were generated using the default t-test hypothesis testing over biological replicates [43].

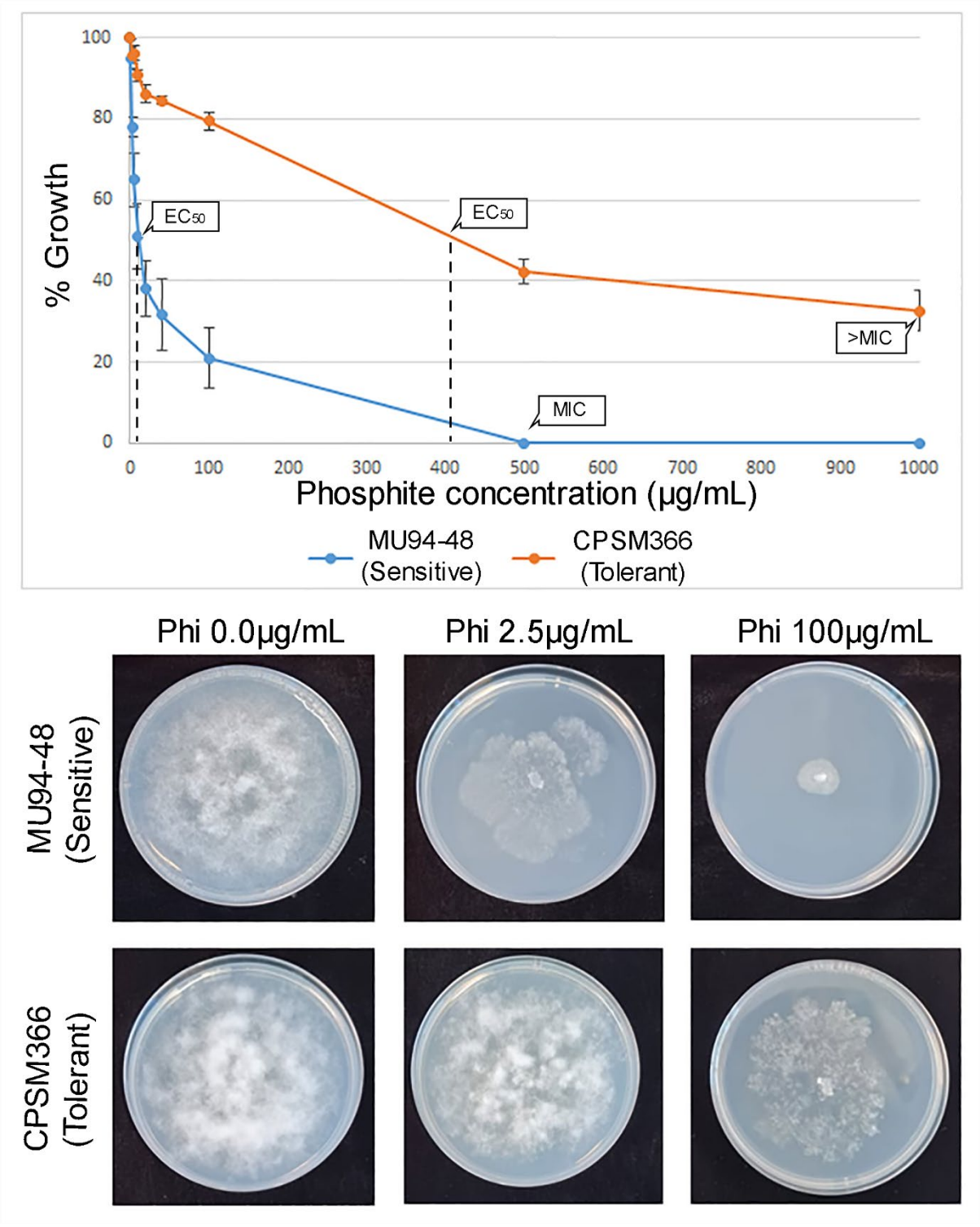
To elucidate the functions of the detected proteins and understand the biochemical differentiation between samples, Gene Ontology (GO), KEGG and Interpro were used [47–49]. For qualitative analysis, a Fisher's exact test was performed on assigned GOs to indicate GO enrichment within samples compared to assigned ontologies of the whole genome annotation in each respective organism.

3 RESULTS

3.1 Growth inhibition of *P. cinnamomi* isolates by phosphite

To determine the sensitivity of *P. cinnamomi* isolates MU94-48 and CPSM366 to phosphite, both isolates were grown on phosphite-supplemented media, and sensitivity was determined based on the radial growth of hyphal colonies. Both isolates exhibited a similar radial growth rate in the absence of phosphite (Figure 1). The MIC for the sensitive and tolerant isolates were 500 µg/ mL and >1000 µg/ mL respectively. At concentrations of 1000 µg/ mL the growth of CPSM366 was reduced but not completely inhibited. The EC_{50} for the MU94-48 and CPSM366 were 10.8 µg/ mL and 415.6 µg/ mL respectively.

For proteomic analyses, 0.0 µg/ mL and 2.5 µg/ mL were used as these represent an untreated and sub-lethal dose of phosphite resulting in reduced growth of 20% and 4% for MU94-48 and CPSM366, respectively.



178

179 **Figure 1.** *In vitro* growth inhibition of MU94-48 and a CPSM366 in response to phosphite treatment
180 indicating the MIC and EC_{50} for each isolate. Images of mycelial growth on the phosphite supplemented
181 media are also displayed.

182

183

3.2 Phosphite induced significant alterations in the proteome of MU94-48 and CPSM366.

To determine the biochemical differences between MU94-48 and CPSM366 treated with phosphite, the soluble intracellular proteome of each isolate was compared by qualitative and quantitative proteomic analyses. 1393 proteins were unique to MU94-48, 280 were unique to the CPSM366 and 1572 were common between the two isolates (Figure 2). Of the common proteins, 171 were higher in abundance in MU94-48 compared to CPSM699 and 90 were lower in abundance. 1311 were not differentially abundant. Overall, there was a significantly higher number of proteins identified in MU94-48, with 42.9% only observed in this isolate.

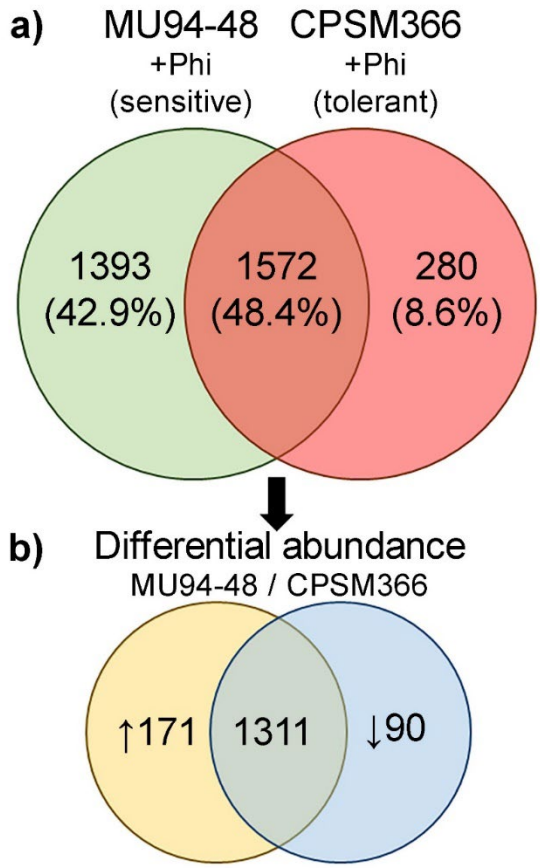


Figure 2. Protein identification in phosphite treated MU94-48 and CPSM366. **a)** Indicating the total number of unique and common proteins between the sensitive and tolerant isolates. **b)** Differential abundance of the common proteins by label-free quantification shows the increased and decreased abundance of proteins in phosphite-treated MU94-48 compared to phosphite-treated CPSM366. The number of proteins identified in all samples obtained for the *in vitro* assay are shown in Supplementary material 1.

3.3 Phosphite induces a stress response in MU94-48.

GO enrichment analysis and differential protein abundance between the MU94-48 and CPSM366 were used to elucidate biochemical changes from phosphite treatment (Figure 3). In MU94-48, an enrichment of putative stress response proteins was observed including glutathione S- transferases (GST), thioredoxins, peptidases, proteasomes and proteolytic enzymes [50]. These indicate that the organism is under stress in these conditions. Putative stress proteins were not observed in CPSM366.

3.4 An increase in signalling in response to phosphite treatment

Enrichment of GOs that are associated with protein signalling including phosphorylation (eg. ATPases, GTPases, PKs), and ubiquitination were observed [51] (Figure 3). GOs that are associated with positive regulation of DNA damage were enriched in addition to tRNA binding, DNA binding, tRNA methylation and mRNA splicing and processing. Inositol signalling and phosphorylation were significantly lower in abundance in MU94-48 coinciding with the enrichment of phosphatidylinositol binding in CPSM366. Positive regulation of TORC1 signalling and Wnt transmembrane signalling GOs were enriched. This indicates stress response signalling, increase in gene expression to compensate for the loss of biological material, and signalling to coordinate cellular processes during stress conditions [52,53].

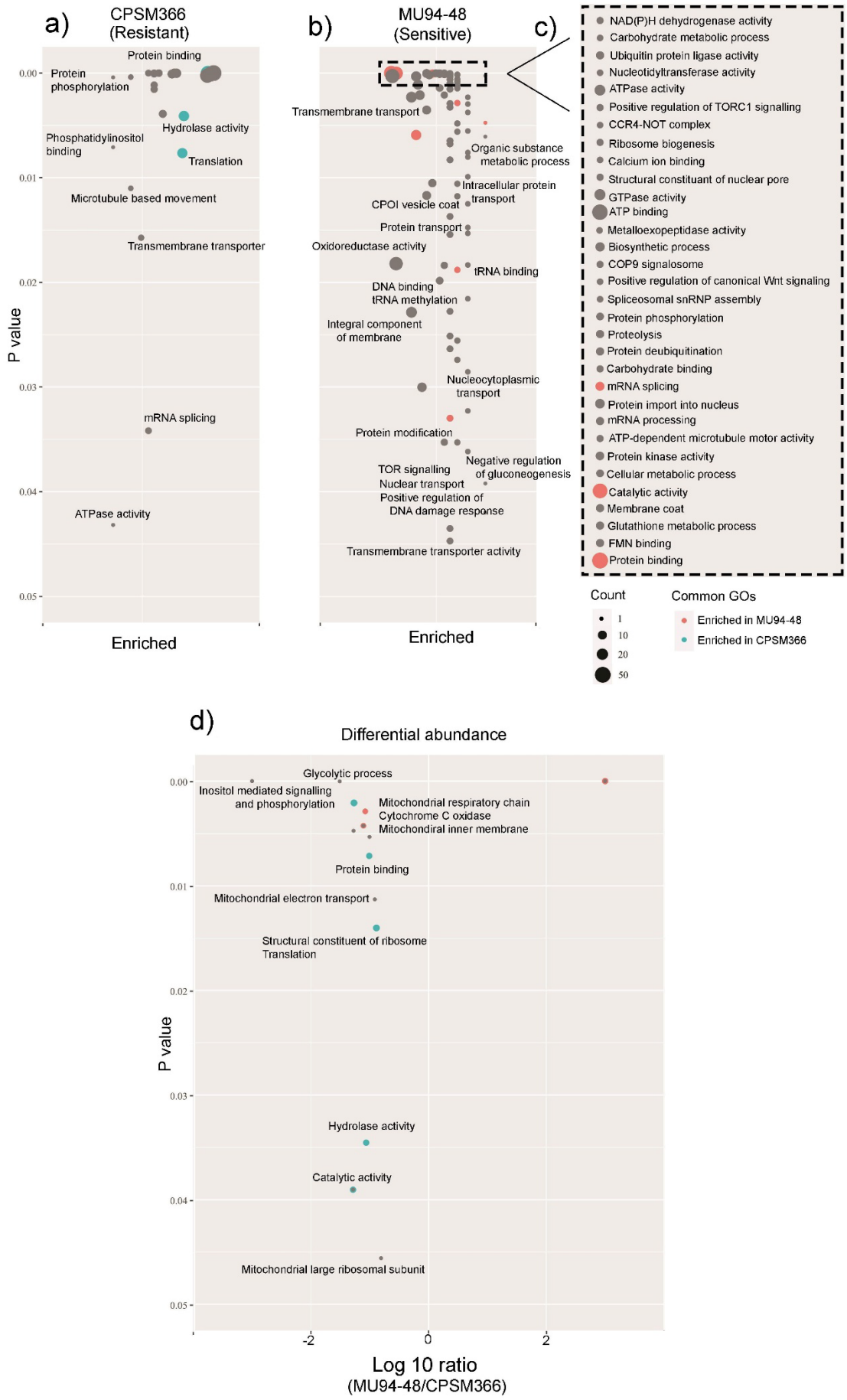
3.5 More transporters in MU94-48 than CPSM366 when treated with phopshite.

A higher diversity of transporters was found in MU94-48, including transmembrane transporters, and intracellular transporters, along with facilitators of transport such as COPI vesicle coatomer proteins, COP9 signalosome, armadillo-like proteins and clathrin proteins (Figure 3). MU94-48 may be using transporters such as ABC and MFS to pump out phosphite as a means of attempted resistance or toxic biproducts of phopshite metabolism to facilitate the high level of signalling to facilitate this process.

3.6 Phosphite alters mitochondrial functionin in phosphite treated MU94-48.

A significantly lower abundance of mitochondrial-associated ontologies were observed in MU94-48 as indicated by mitochondrial respiratory chain, cytochrome C oxidase, mitochondrial electron transport and mitochondrial ribosomal subunit GOs. This could be a result of oxidative stress in MU94-48 or may suggest that similarly to other fungicides, phosphite alters mitochondrial respiration [54,55]. The KEGG ontologies between phosphite-treated MU94-48 and CPSM366 did not show changes in distinct metabolic pathway clusters but rather showed general metabolic disorder (Supplementary material 2). However, the disordered KEGG orthologues in MU94-48 suggest that phosphite is exerting a cytotoxic effect on the sensitive isolate.

We then examined the proteome of MU94-48 and CPSM66 compared to their respective untreated controls to ensure that these observations were not artifacts of the isolates. It was observed that these proteomes were comparable to those between phosphite treated MU94-48 and CPSM366 (Supplementary material 3). This includes the enrichment in putative stress proteins such as oxidoreductase activity, intracellular signalling and protein activation, and gene expression-related ontologies. Additionally, a significant reduction in inositol biosynthesis and signalling, mitochondrial electron transport-related GOs were observed, reflecting the observations between the two phosphite-treated isolates. Similarly, we compared the proteome of untreated and phosphite-treated CPSM366 and found less biochemical responses than when MU94-48 was compared to CPSM366. The full GOs, KEGG orthologues and gene functions for all *in vitro* qualitative and quantitative comparisons are shown in Supplementary Material 4.


a)
CPSM366
(Resistant)
b)
MU94-48
(Sensitive)
c)
P value
Enriched
Protein binding
Protein phosphorylation
Hydrolase activity
Phosphatidylinositol binding
Translation
Microtubule based movement
Transmembrane transporter
mRNA splicing
ATPase activity
Transmembrane transport
Organic substance metabolic process
Intracellular protein transport
CPOI vesicle coat
Protein transport
Oxidoreductase activity
tRNA binding
DNA binding
tRNA methylation
Integral component of membrane
Nucleocytoplasmic transport
Protein modification
Negative regulation of gluconeogenesis
TOR signalling
Nuclear transport
Positive regulation of DNA damage response
Transmembrane transporter activity
NAD(P)H dehydrogenase activity
Carbohydrate metabolic process
Ubiquitin protein ligase activity
Nucleotidyltransferase activity
ATPase activity
Positive regulation of TORC1 signalling
CCR4-NOT complex
Ribosome biogenesis
Calcium ion binding
Structural constituant of nuclear pore
GTPase activity
ATP binding
Metalloexopeptidase activity
Biosynthetic process
COP9 signalosome
Positive regulation of canonical Wnt signaling
Spliceosomal snRNP assembly
Protein phosphorylation
Proteolysis
Protein deubiquitination
Carbohydrate binding
mRNA splicing
Protein import into nucleus
mRNA processing
ATP-dependent microtubule motor activity
Protein kinase activity
Cellular metabolic process
Catalytic activity
Membrane coat
Glutathione metabolic process
FMN binding
Protein binding
Count
1
10
20
50
Common GOs
Enriched in MU94-48
Enriched in CPSM366

d)
Differential abundance
P value
Log 10 ratio
(MU94-48/CPSM366)
Glycolytic process
Inositol mediated signalling and phosphorylation
Mitochondrial respiratory chain
Cytochrome C oxidase
Mitochondiral inner membrane
Protein binding
Mitochondrial electron transport
Structural constituent of ribosome
Translation
Hydrolase activity
Catalytic activity
Mitochondrial large ribosomal subunit

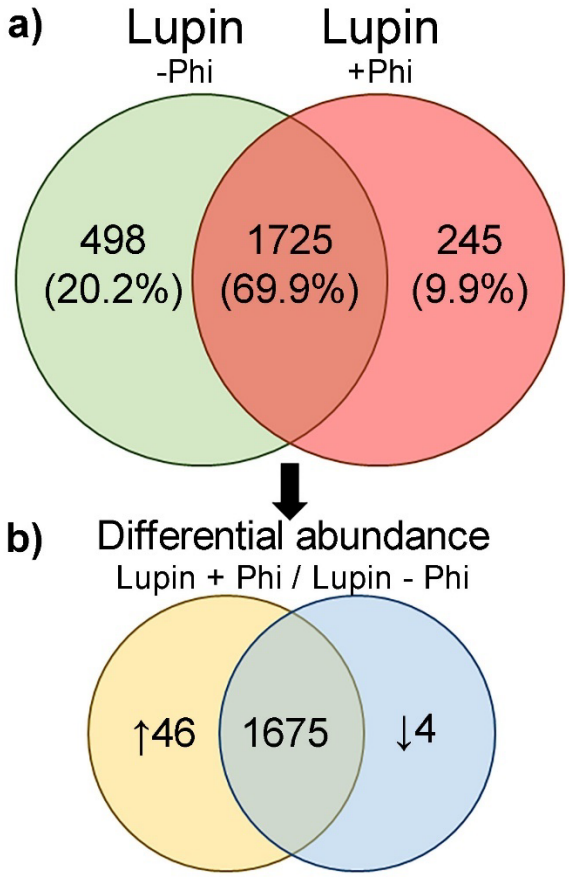
240

241 **Figure 3.** GO enrichment and differential abundance between MU94-48 and CPSM366 treated with
242 phosphite. **a)** and **b)** show GO enrichment in the phosphite treated tolerant and sensitive isolates
243 respectively, where the P value is generated by GO enrichment and each point is separated on a log10
244 scale generated by each GO count to the total GO count for the sample set. These x axis for the
245 enrichment data is an arbitrary space for visualisation purposes only. **c)** The enriched gene ontologies
246 in the sensitive isolate at $P < 0.001$. **d)** Differential abundance of proteins between the sensitive and
247 tolerant isolate. Ratios are generated by the peptide signal of each protein in MU94-48 compared to
248 CPSM366. P values depict the significance of the differential abundance.

249 ### 3.7 Differential abundance of proteins between untreated and phosphite treated *Lupinus angustifolius*.

250 We hypothesise that phosphite alters the biochemistry in plant physiology by facilitating plant immunity
251 to prevent infection by phytopathogens such as *P. cinnamomi* . To investigate this, the proteome of *L.*
252 *angustifolius* treated with and without phosphite were compared. 498 proteins were unique in the
253 untreated Lupin sample and 1725 were unique in the phosphite-treated Lupin (Figure 4). 245 proteins
254 were common between the untreated and treated lupin samples of which 46 were significantly higher
255 in abundance when phosphite was applied and 4 were significantly lower.

256



257

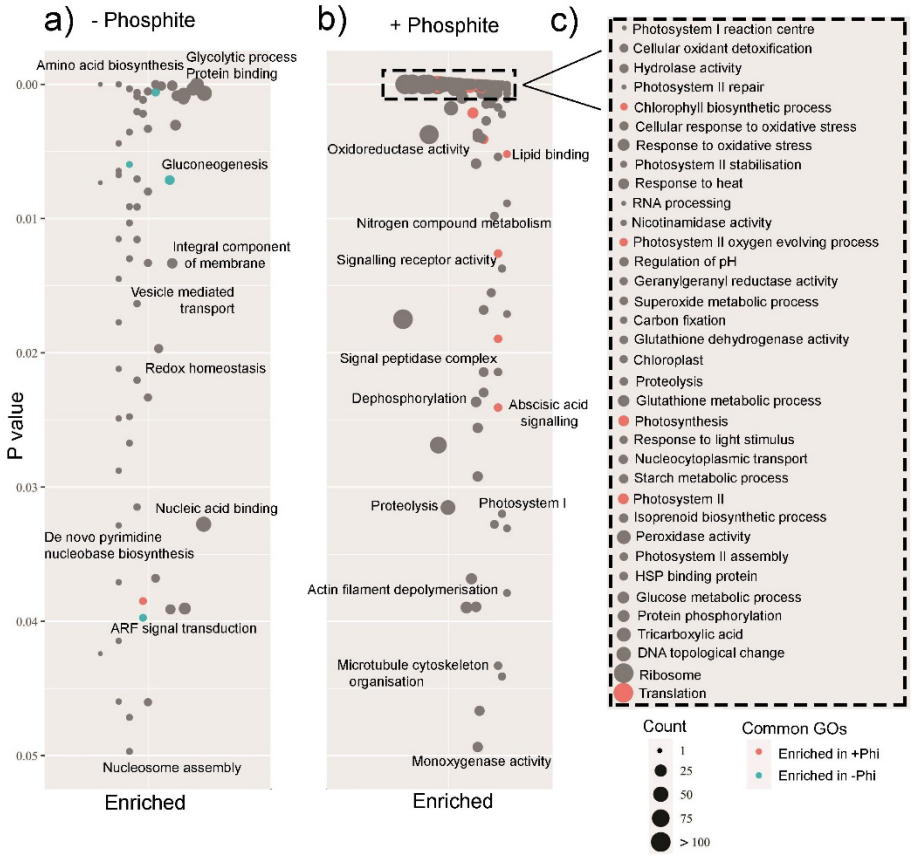
258 **Figure 4**. Protein identification between untreated and phosphite treated lupins. **a)** Indicating the total
259 number of unique and common proteins between the untreated and phosphite-treated lupin. **b)**
260 Differential abundance of the common proteins by label-free quantification shows the increased and
261 decreased abundance of proteins in phosphite-treated lupin compared to the untreated control. The
262 number of proteins identified in all samples obtained for the *in planta* assay are shown in
263 Supplementary material 1.

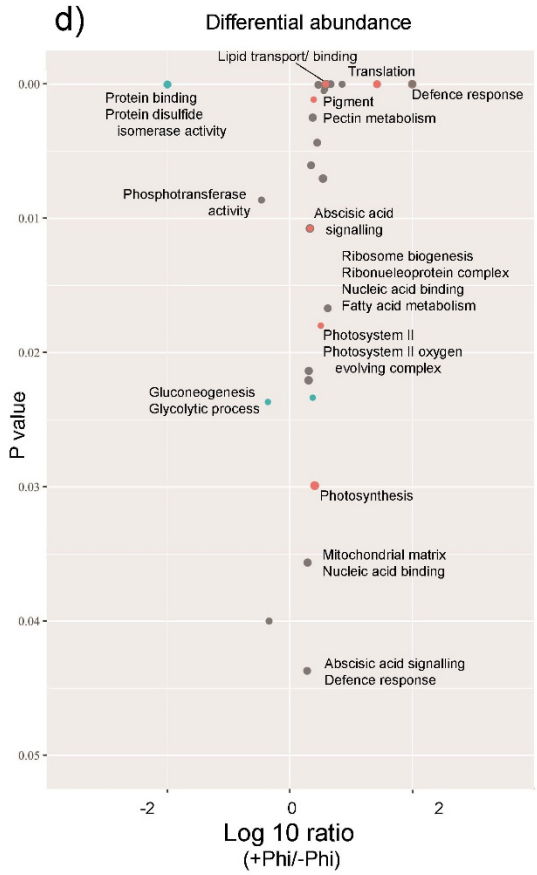
264 **3.8 Phosphite increased the abundance of photosynthetic and carbon fixation proteins in *L.***
265 ***angustifolius*.**

266 GO enrichment and KEGG pathway enrichment analyses were used to examine biochemical effects in
267 lupin treated with phosphite, (Figure 5 and 6). It was observed that GOs containing proteins that are
268 associated with photosynthesis and starch metabolism were significantly enriched in phosphite-treated
269 lupins. Photosynthesis, photosystem units, geranylgeranyl reductase, chlorophyll biosynthesis, carbon
270 fixation and TCA cycle were all enriched in phosphite-treated lupins. Clustering of KEGG ontologies only
271 found in the phosphite-treated lupin mapped to photorespiration, photosynthesis, reductive pentose
272 phosphate cycle, isoprenoid biosynthesis and carbon fixation (Figure 6). As lupin seedlings were grown

under artificial light with roots exposed leading to the development of photosynthetic-capable tissues, these were excised as part of the excised root tip for protein extraction (Figure 7).

Proteins associated with glucose, starch, and lipid metabolism GOs were enriched in the phosphite-treated lupin along with related KEGGs such as starch and sucrose metabolism, beta-oxidation and fatty elongation and lactosylceramide. Gluconeogenesis was reduced in abundance which supports the utilisation of carbohydrates and sugars as an energy source to fuel the heightened metabolism. Similarly, KEGG orthologues that are associated with lipid metabolism were also observed as indicated by the clustering of identified KEGG pathways only found in the phosphite-treated lupin (Figure 6). Phosphite treatment results in increased abundance of proteins that are associated with photosynthesis, carbon metabolism and energy production in lupin.


a) - Phosphite
b) + Phosphite
c)
Amino acid biosynthesis
Glycolytic process
Protein binding
Gluconeogenesis
Integral component of membrane
Vesicle mediated transport
Redox homeostasis
Nucleic acid binding
De novo pyrimidine nucleobase biosynthesis
ARF signal transduction
Nucleosome assembly
P value
Enriched
Oxidoreductase activity
Lipid binding
Nitrogen compound metabolism
Signalling receptor activity
Signal peptidase complex
Dephosphorylation
Abscisic acid signalling
Proteolysis
Photosystem I
Actin filament depolymerisation
Microtubule cytoskeleton organisation
Monoxygenase activity
Enriched
Photosystem I reaction centre
Cellular oxidant detoxification
Hydrolase activity
Photosystem II repair
Chlorophyll biosynthetic process
Cellular response to oxidative stress
Response to oxidative stress
Photosystem II stabilisation
Response to heat
RNA processing
Nicotinamidase activity
Photosystem II oxygen evolving process
Regulation of pH
Geranylgeranyl reductase activity
Superoxide metabolic process
Carbon fixation
Glutathione dehydrogenase activity
Chloroplast
Proteolysis
Glutathione metabolic process
Photosynthesis
Response to light stimulus
Nucleocytoplasmic transport
Starch metabolic process
Photosystem II
Isoprenoid biosynthetic process
Peroxidase activity
Photosystem II assembly
HSP binding protein
Glucose metabolic process
Protein phosphorylation
Tricarboxylic acid
DNA topological change
Ribosome
Translation
Count
1
25
50
75
> 100
Common GOs
Enriched in +Phi
Enriched in -Phi


d) Differential abundance
Lipid transport/ binding
Translation
Defence response
Protein binding
Protein disulfide isomerase activity
Pigment
Pectin metabolism
Phosphotransferase activity
Abscisic acid signalling
Ribosome biogenesis
Ribonueleoprotein complex
Nucleic acid binding
Fatty acid metabolism
Photosystem II
Photosystem II oxygen evolving complex
Gluconeogenesis
Glycolytic process
Photosynthesis
Mitochondrial matrix
Nucleic acid binding
Abscisic acid signalling
Defence response
P value
Log 10 ratio
(+Phi/-Phi)

284

Figure 5. GO enrichment and differential abundance between untreated and phosphite-treated lupin. a) and b) show GO enrichment in the untreated and phosphite-treated lupin respectively, where the P value is generated by GO enrichment and each point is separated on a log10 scale generated by each GO count to the total GO count for the sample set. c) The enriched gene ontologies in the phosphite-treated lupin at $P < 0.001$. d) differential abundance of proteins between the untreated and treated lupin. Ratios are generated by the peptide signal of treated lupin compared to the untreated control. P values depict the significance of the differential abundance.

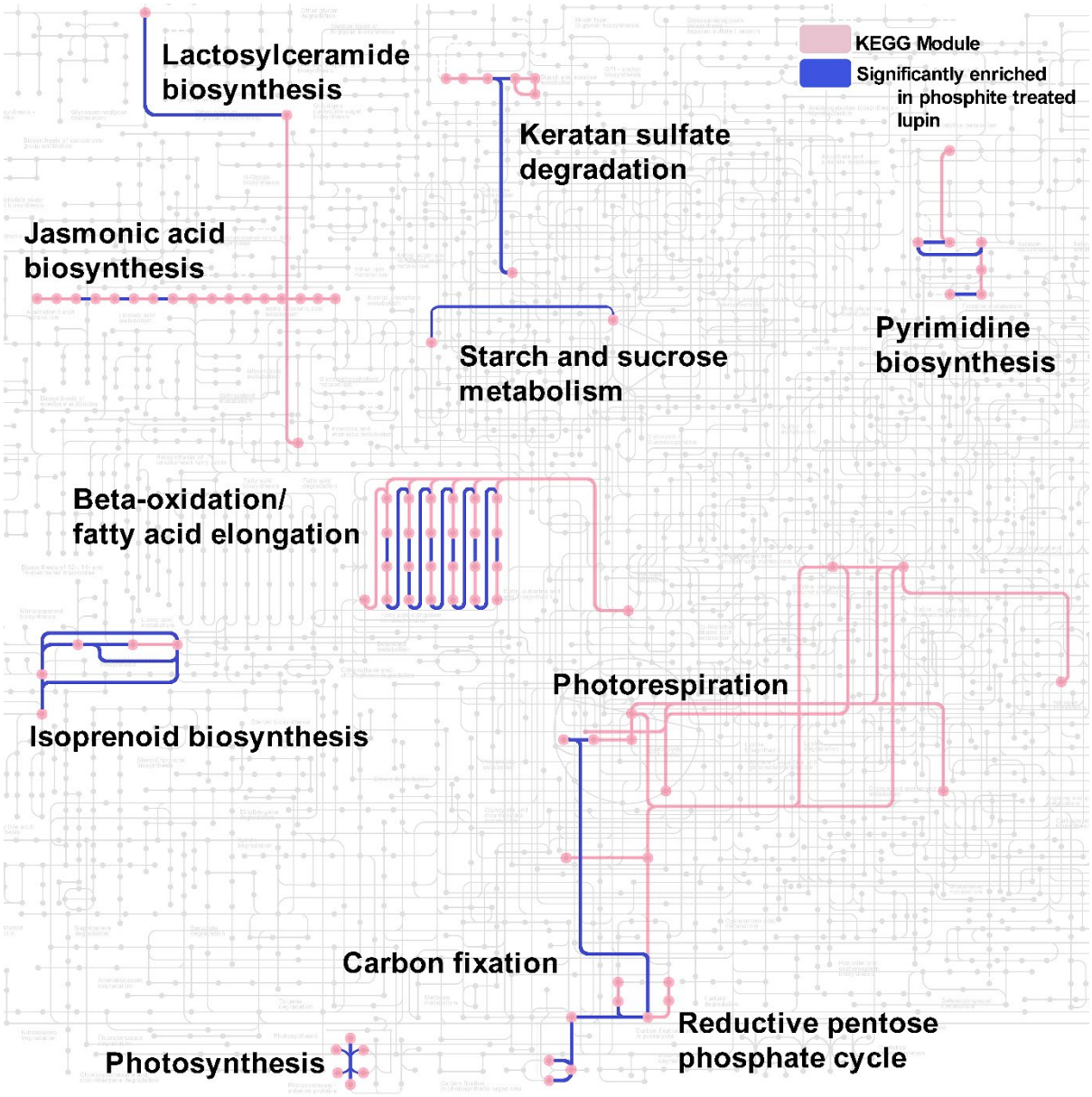
3.9 Transcriptional activities in phosphite-treated *L. angustifolius* are enhanced.

As a complement to the abundance of photosynthetic and metabolic gene ontologies, gene expression was also overrepresented. Translation, ribosomal proteins, DNA topological change, and RNA processing were all enriched in the phosphite treatment (Figure 5). This is also shown by the clustering of KEGG pathways in the phosphite-treated lupin including pyrimidine biosynthesis and keratan sulfate degradation (Figure 6). Hence, an increase in gene expression and biosynthesis accompanies massive physiological and metabolic changes.

3.10 Phosphite triggers the accumulation of defence-related proteins in lupin

Phosphite has previously been reported to increase the production of defence-related molecules in plants, particularly salicylic and jasmonic acid [7,8,56]. An enrichment of defence-related GOs was observed in the phosphite-treated lupin. These included proteolysis, oxidoreductase activity, hydrolases, cellular oxidant detoxification and response to oxidative stress (Figure 5). Secretory peroxidases, superoxide metabolism, abscisic acid signalling, and defence response GOs encompassing genes such as steroid chaperones, programmed cell death and apoptosis were also enriched. Several secondary metabolites were enriched including nicotinamidase and isochorismatase were also found only in the phosphite-treated lupin.

Precursors to the salicylic acid (SA) pathway including isochorismatase and actin depolymerisation GOs were enriched in the phosphite-treated lupin. KEGG analysis revealed components associated with the jasmonic acid (JA) pathway were only found in the phosphite-treated lupin. This shows that more defence-related proteins including secondary metabolites were enriched and higher in abundance in the phosphite-treated lupin.



315

316 **Figure 6.** A representative set of the KEGG pathways enriched in the phosphite-treated lupin obtained
317 from both the qualitative and quantitative data set. Each dot represents a KEGG entry, and each line
318 represents individual KEGG ontology identifiers. KEGG entries that form clear pathway sets from
319 overrepresented modules are labelled. The proteins that are assigned to these KEGG entries are shown
320 in Supplementary material 5.

321 **3.11 The effect of phosphite on MU94-48 and CPSM366 isolates during host infection.**

322 To test the effect of phosphite during host infection, *L. angustifolius* was treated with phosphite and
323 subsequently inoculated with the sensitive or tolerant isolate of *P. cinnamomi* (Figure 7). Untreated
324 lupin infected with MU94-48 and CPSM366 developed comparable lesions. When phosphite-treated *L.*
325 *angustifolius* was infected with MU94-38 no lesion was observed showing sensitivity to phosphite *in*

planta. When inoculated with CPSM366, there was no reduction in lesion score compared to the untreated lupin.

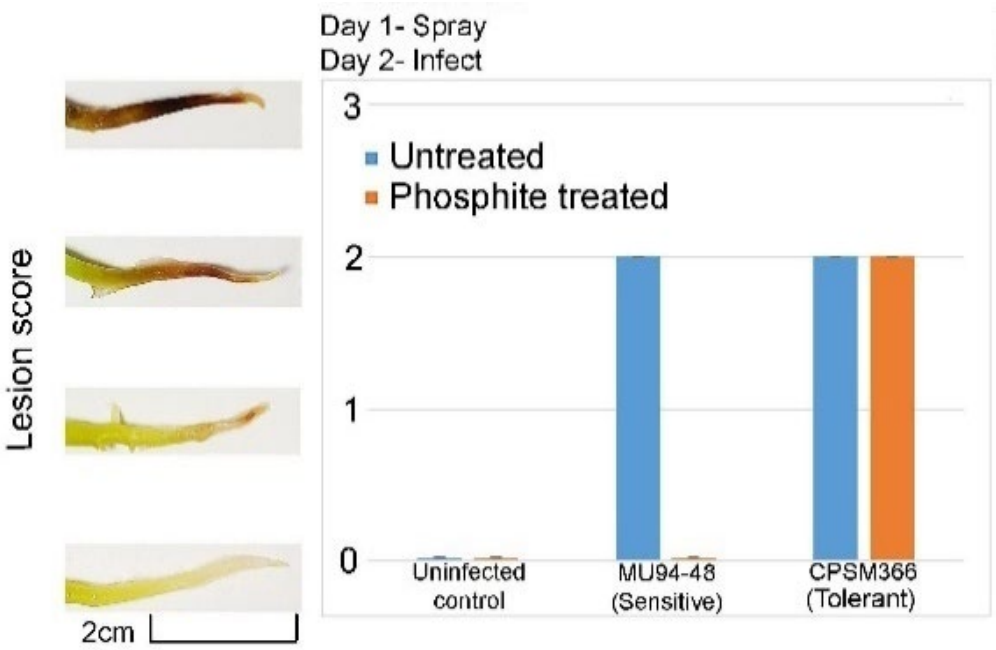


Figure 7. Lesion scores of lupin roots when treated with phosphite and infected with the two isolates of *P. cinnamomi*. Lesion scores were taken on day 3.

4 DISCUSSION

The mode of action of phosphite in *Phythophthora* pathosystems is a central question in the development of future management strategies. As resistance to the only effective chemical for the control of *P. cinnamomi* and some other ooomycetes such as downy mildews emerges, the pressure to find resistance genes or alternative management strategies mounts. An understanding of the mode of action of phosphite could aid in the development of better oomyceticides, which in turn would contribute to improving existing management strategies. Previous literature has suggested both direct and indirect mechanisms of phosphite on the pathogen and its host plants however the biochemical mechanisms in which these occur have not been defined. Our study aims to deconvolute the biochemistry of this system and gain a clearer insight into the pathways altered by phosphite. *L. angustifolius* (lupin) is used as a model as it is susceptible to *P. cinnamomi* infection, it has a published genome sequence required for proteomics work, and it grows rapidly compared to native and horticultural *P. cinnamomi* hosts [45,57].

The *in vitro* growth assay demonstrates that phosphite has a direct inhibitory effect on mycelial growth in *P. cinnamomi*. The EC_{50} of previously reported phosphite-sensitive *P. cinnamomi* isolates ranges between 4 µg mL^{-1} and 25 µg mL^{-1} [21,58]. The EC_{50} MU94-48 falls within this range and can be considered

highly sensitive to phosphite. Reported tolerant isolates of *P. cinnamomi* have EC_{50} concentrations up to 150 µg mL^{-1} and other species of *Phytophthora* have EC_{50} values up to 350 µg mL^{-1} , of which CMSP366 exceeds [21,58–60]. Isolates of two *Phytophthora spp, P. citrophthora* and *P. syringae* were screened for sensitivity to phosphite were considered sensitive when their EC_{50} values were below 25 µg mL^{-1}, whereas *P. nicotianae* isolates with EC_{50} values above 75 µg mL^{-1} were considered moderately resistant or resistant [25]. CPSM366 is therefore considered as highly resistant to phosphite, highlighting the need for understanding its biochemistry and the development of alternative management strategies.

A proteomic approach was taken to understand how phosphite interacts with the two *P. cinnamomi* isolates MU94-48 and CPSM366 and causes growth reduction. The data obtained from this work was used to build a model suggesting the possible mechanisms in which phosphite directly affects the pathogen (Figure 8). In the presence of phosphite, an abundance of putative stress proteins is observed in the sensitive isolate (Figure 8a). The pathogen expresses GSTs and thioredoxins as a detoxifying response to the influx of phosphite [61]. Hydrolase, proteolysis and peptidase proteins could also be produced by the pathogen in this case for nutrient recycling, detoxification of xenobiotics, or could be products of cell death caused by phosphite [50,62]. Extensive putative stress response proteins were only observed in MU94-48. This indicates that CPSM366 may have mechanisms to better cope with the effects of phosphite.

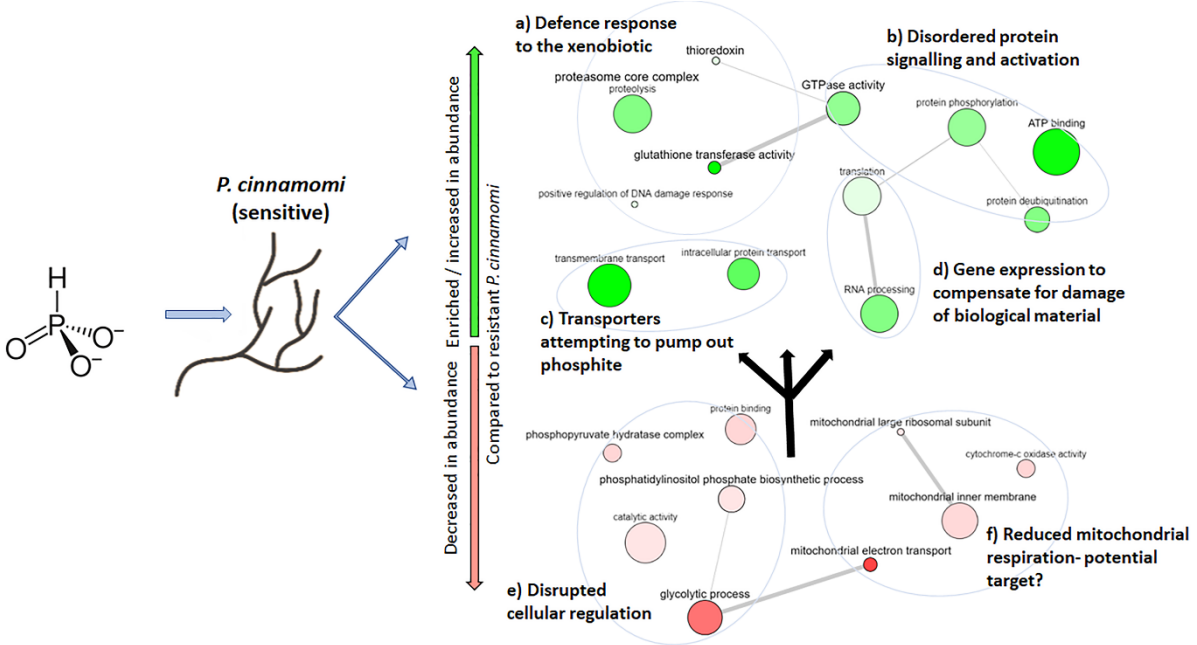


Figure 8- A proposed model on the direct effect of phosphite on *P. cinnamomi* MU94-48 from the combination of qualitative and quantitative comparisons between MU94-48 and CPSM366. Black arrows indicate a possible cascading effect of core biochemical pathways that are disrupted as a result

of phosphite treatment. The size of bubbles represents enrichment relative to each other for visualisation.

Signalling molecules related to stress response were also induced along with the disruption of the regulation of cellular processes caused by the exposure of MU94-48 to phosphite (Figure 8b). Several core regulators of cell growth, metabolism and signalling were altered as a result of phosphite treatment. For example, TORCI signalling was enriched in MU94-48, which is involved in aspects of cell growth and metabolism [53]. Positive response to regulation of DNA damage and increase in gene expression-related ontologies shows that phosphite could cause DNA damage and subsequent increase in gene expression to compensate for the loss of DNA, proteins and cell material through biosynthesis (Figure 8d) [63]. These mechanisms of coping with extensive damage do not seem to be effective enough to protect the sensitive isolate from damage by phosphite.

Greater diversity of membrane transporters were observed in the proteome of MU94-48 than in CPSM366 (Figure 8c). These transporters include ABC and MSF-type, which have been described to participate in the removal of xenobiotics from the cell [64,65]. This may be applied in MU94-48 to increase to export of phosphite out of the cell or increase protein translocation that facilitate detoxification. Additionally, facilitators of intracellular transport were also enriched in MU94-48 suggesting that, unlike CPSM366, MU94-48 attempts to remove the toxic xenobiotic.

Compared to the tolerant isolate CPSM366, several constituents of the mitochondrial respiratory pathway were reduced in abundance in the sensitive isolate (Figure 8f). In fungal pathogens, programmed cell death can be triggered by mitochondrial-initiated signalling that can be activated by cell damage, exposure to toxic xenobiotics and oxidative stress [66]. During these stress conditions, reactive oxygen species can disrupt components of the mitochondrial respiration chain causing subsequent cell death [67–69]. As this is a core pathway for cellular function, mitochondrial respiration is commonly used as a target for chemical control of fungal phytopathogens by single-site fungicides [55,70]. For example, strobilurins are a broad-spectrum class of fungicide applied to control fungal crop diseases. Strobilurin binds cytochrome b complex III, inhibiting mitochondrial respiration [71]. Similarly, azoxystrobin blocks electron transport in mitochondrial respiration by blocking electron transport [71,72,73]. Evidence presented here might suggest a broader inhibitory activity in the case of phosphite as it seems to be affecting the pathogen's metabolism at multiple levels. Many fungicides have multi-site modes of action, where several biochemical processes are disrupted such as chlorothalonil, folpet, thiram, sulphur and copper [74,75].

The metabolic, signalling, regulatory and stress responses were not observed in CPSM366. If phosphite acts as a multi-site oomyceticide, CPSM366 has likely developed physiological adaptation to tolerate phosphite such as efflux, detoxification or alteration in influx transporters [76,77].

It has been suggested that phosphite alters the plant system to better cope with potential attacks from oomycete pathogens by priming the plant immune system [7,8]. In addition, phosphite has been reported to act as a biostimulant in plants [78]. A proteomic approach was used to obtain a detailed biochemical snapshot of the proteins differentially abundant in phosphite-treated *L. angustifolius.* The proteomic data obtained from the *in planta* assay was used to generate a model that describes how phosphite alters plant metabolism and induces an increase in defence-related proteins (Figure 9).

The biochemical differentiation between phosphite treated and untreated *L. angustifolius* suggests an increase in metabolic activity. Constituents of the photosynthetic process, carbon fixation and citric acid cycle suggest that phosphite is driving metabolism in plants (Figure 9a). In addition, an increase in lipid and carbohydrate metabolism was observed, indicating that stored carbohydrates can be metabolised for energy generation (Figure 9b). The KEGG orthologue reductive pentose phosphate cycle along with the significant decrease in gluconeogenesis in the phosphite-treated lupin highlighting that sugars are utilised for energy production in this state, not stored [79].

An increased metabolism has been observed in other phosphite-treated plants compared to untreated controls along with its biostimulant effect [23,78,80,81]. The application of phosphite improves yield, biomass, fruiting and growth, with a field trial applying phosphite in avocado plantations showing a significant increase in fruit production [82,83]. In potato, phosphite application resulted in reduced seedling emergence time, increased leaf size and biomass [84]. This 'greening effect' has been demonstrated amongst other fungicides in agriculture, where yield, biomass, leaf surface area, and protein content are increased as a result of fungicide application [85–87]. The response observed in the phosphite-treated lupin seedlings is similar to the greening effect, where phosphite boosts the metabolism of the plant. Non-leaf plant structures such as roots, stems, flowers and seeds have photosynthetic potential when exposed to light and carbon fixation has been reported to occur in many plant roots along with green roots [88–90].

Defence-related gene expression has been demonstrated in several phosphite-treated crops. In potato, phosphite treatment caused a significant increase in transcription of salicylic and jasmonic acid [8]. Proteomic analysis of a similar system showed an increase in the abundance of peroxidases, glutathione S-transferase and proteinase inhibitors [91]. In the present study, enrichment of defence and stress-related proteins in *L. angustifolius* were detected as a result of phosphite treatment, which may act in favour of the defence response of the plant.

Secretory peroxidases were also enriched in the phosphite-treated lupin which can oxidise toxic compounds and have functional roles in defence and biosynthesis [92]. Superoxide metabolism and phospholipid binding composed of annexin genes were also enriched. Superoxide dismutase is used in plant defence against reactive oxygen species [93,94]. Isoprenoids identified in the phosphite-treated lupin are not only carriers in photosynthetic and respiratory electron transport and have additional functions as antioxidants [95]. Defence-related ontologies with gene functions related to steroid chaperoning indicating programmed cell death and apoptosis were also abundant [96]. Signalling and binding of abscisic acid, a key hormone involved in signalling during stress and defence response to abiotic and pathogens was significantly higher in abundance in the phosphite-treated lupin. Nicotinamidase and isochorismatase are involved in plant growth, hydrolase activity and synthesis of salicylic acid [97–100] JA pathway is associated with defence response against necrotrophic microbial pathogens and abiotic stresses and the SA pathway is involved with biotic stressors, cell death and hypersensitive responses in plants [101,102].

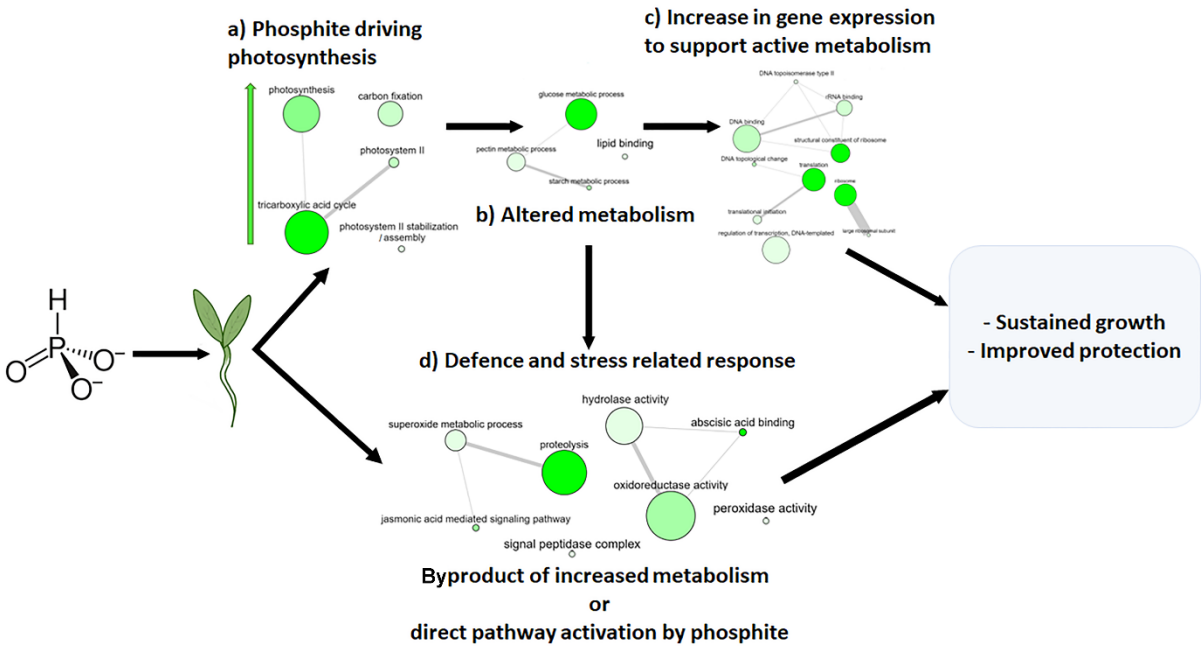


Figure 9- A proposed model on the effects of phosphite on *L. angustifolius* from the combination of qualitative and quantitative proteome data between the untreated and treated lupin.

In this system, phosphite is driving both metabolism and defence by directly promoting their biochemical pathways [8]. The production of stress molecules such as abscisic acid can at the same time be used as signalling molecules to trigger plant immunity [103]. The production of defence and stress-related molecules in the plant could also be acting in response to phosphite as a xenobiotic substance [104,105]. In the field, the ideal use of phosphite is part of a preventative strategy for the management of *P. cinnamomi* infection [11,13,14]. The *in planta* assay demonstrates that phosphite application does not

reduce the observed lesion when lupin plants are infected with the tolerant isolate. If the elevated defence response impacts the colonisation ability of *P. cinnamomi,* it is not observed in this system. Potentially the induction of defense molecules by phosphite in this system may not have reached a sufficient amplitude for host resistance.

5 CONCLUSION

Our data present a new perspective on the mode of action of phosphite. We have provided evidence to propose models of the direct and indirect mode of action of phosphite. This data demonstrates a comprehensive snapshot of the metabolic dysregulation in the sensitive isolate when treated with phosphite, suggesting it alters one or more core biochemical pathways, may inhibit these pathways or be products of efflux and detoxification. The tolerant isolate is likely to have adapted to block phosphite from entering the cell, or if phosphite targets constituents of mitochondrial respiration, adapted to block this interaction. Our model proposes an alternative avenue of plant responses to phosphite, where phosphite drives multiple biochemical pathways and as a byproduct, more defence-related proteins are produced. We proposed probable mechanisms based on proteomic data that indicates a bi-modal mode of action of phosphite on both the pathogen and the host plant. Further studies are required to replicate this experimentation in a native or crop context where host plants are grown in soil, injected with phosphite and subsequently soil inoculated with *P. cinnamomi* to determine if these results are comparable in the field. Systemic changes in phosphite-treated *L. angustifolius* should be determined such as changes in photosynthesis and respiration in leaves to confirm the current observations. Additionally the accumulation of phosphite in the plant tissue and subsequent uptake by the pathogen cannot be excluded. The outcome of this study presented opportunities for functional validation to determine the phosphite mode of action in crop protection.

Availability of data and materials

Spectral data used for this study are available at Figshare (DOI:10.6084/m9.figshare.20026214).

Acknowledgements

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cinnamomi isolates. We also thank Mr Leon Lenzo for his critical feedback on the manuscript and Dr Paula Moolhuijzen for her input into bioinformatic visualisation.

Supplementary material 1- Protein identification of *in vitro phosphite* treated MU94-48 and CPSM366 treated with phosphite and of the phosphite treated lupin roots. FDR was set to <1%.

Supplementary material 2- Th KEGG pathways identified from the protenis only found in MU94-48. Green lines indicate the KEGG pathways assigned to the unique proteins in phopshite treated MU94-48 compared to the untreated control. There are no clear clusters of pathways in this sample indicating that phosphite affects multiple processes in the sensitive isolate.

Supplementary material 3- Representative GOs obtained from phosphite treated MU94-48 and CPSM366 compared to their respective untreated controls.. **a)** Enriched GOs in the proteins uniquely identidied in untreated MU94-48 compared to treated MU94-48. **b)** GOs associates with the differentially abundant proteins between untreated and phopshite treated MU94-48. **c)** Enriched GOs in the proteins uniquely identidied in treated MU94-48 compared to tnreated MU94-48. **d)** Enriched GOs in the proteins uniquely identidied in untreated CPSM366 compared to treated CPSM366. **e)** GOs associates with the differentially abundant proteins between untreated and phopshite treated CPSM366. **f)** Enriched GOs in the proteins uniquely identidied in treated MU94-48 compared to tnreated MU94-48. Green bubbles in **a), c), d)** and **f)** indicate significantly enriched GOs, green bubbles in **b)** and **e)** indicated significantly higher in abundance in the treated samples and red bubbles indicate significantly lower abundance in the treated samples compared to untreated samples.

Supplementary material 4- The full list of GOs, Interpro and KEGGs between the untreated and phosphite treated qualitative and quantitative *in vitro* data set.

Supplementary material 5- The full list of GOs, Interpro and KEGGs between the untreated and phosphite treated qualitative and quantitative *in planta* data set.

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Chapter 6 General discussion and future perspective

6.1 Improving oomycete genome annotations

The knowledge of the molecular aspects of *P. cinnamomi* biochemistry and virulence is lacking compared to many other oomycetes such as *P. infestans*. The project aimed to gain a better understanding of the pathogenicity of *P. cinnamomi* and create a foundation that can be used for subsequent research. Pre-emptive consideration of the resources required for high-confidence proteomic work led us to examine the quality of the draft genome currently available for *P. cinnamomi.* Compared to other *Phytophthora spp,* this genome had an unusually higher number of annotated genes. As shotgun proteomics is reliant on accurately annotated genomes, this posed the first hypothesis of this thesis (Chapter 1).

Using a representative subset of cellular proteomes and robust mass spectra, I demonstrated that a proteogenomic pipeline can be applied to oomycete pathogens to improve genome annotation. These methods facilitated the correction of 60 genes from the draft genome and the curation of 23 new genes with the support of peptide data (Andronis et al., 2021). These changes account for 3% of the detected proteome. This significant improvement in the annotation not only reinforces the potential for missed gene models through traditional means of annotation but also highlights the potential for genes with unsuspected features that may be missed across all phyla.

Proteogenomic methods used in Chapter 3 relied on high-confidence peptides, as well as a well-sequenced genome and significant BlastP matched to other *Phytophthora* species. The main drawback here is that the manual curation of these genes requires significant BlastP hits. Since these genes were elusive to typical gene calling methods, then they are less likely to return significant results. This leaves the potential for hundreds to thousands of new genes that cannot be curated in this manner which may have the potential to identify biologically important genes. Curating such genes without additional support would introduce different aspects of error such as incorrect gene boundaries and reading frames.

However, perhaps these candidates could be validated using other methods. If implemented into *in silico* annotation methods, more accurate and novel gene models will be available for downstream application such as virulence gene identification and effector discovery. This newly improved genome has provided a stronger foundation of predicted proteins and is now available for further proteomic and genomic work. This version 2 of the genome was used for all subsequent work in Chapter 4 and Chapter 5.

Another advantage of using mass spectral data to support gene annotation is the depth in which protein products can rapidly alter in response to subtle environmental changes. RNA seq can be used to identify transcripts expressed in different states or treatments but there are some major limitations to using transcripts for gene annotation. It is well known that transcript and proteome abundances poorly correlate (Lu et al., 2006; Wright et al., 2012). The differences stem from several factors including RNA secondary structures, regulatory proteins, regulatory sRNAs, coding biases, ribosomal density, protein half-lives, and post-translational modifications (Maier et al., 2009). Although transcripts are extremely useful in elucidating gene expression, its application on informing on its protein products are more ambiguous due to the transcript-protein discrepancy. Proteome data therefore is more relevant in this context as it is a more accurate representation of the biology of the organism as it is at the protein level, i.e., the functional level. It provides with the opportunity to unravel not only qualitative data but differential abundance of proteins. Proteomics (like all other omic methods) does however have its own limitations. It may be a more accurate representation of the organisms biochemistry but detection is limited to a few thousand proteins. Oomycete pathogens have a total predicted gene count of 15,000-30,000 genes, so detection of proteomes by proteomics is potentially missing a large portion of gene models. Therefore an integrated approach using both transcripts and proteomics is ideal for gene annotation. Additionally, many proteins require post-translational modification to be active or require other interacting proteins to become functional. These cannot be easily elucidated using shotgun proteomic techniques. In the datasets used in Chapters 3, 4 and 5, there were several

proteins that did not have assigned putative function. If the protein function is not known, then there is a gap in the data of potentially biologically relevant proteins.

The draft genome of *P. cinnamomi* MU94-48 was generated in 2010, therefore re-sequencing the genome using up-to-date current technology may be necessary to obtain a more accurate genome and subsequent gene prediction. This may also resolve unmapped regions where longer reads (eg PacBio or Nanopore) would assist in more accurate annotation and areas with poor coverage. With the assistance of the currently available mass spectra from this study, the genome annotation will be a more accurate representation of the functional proteome.

6.2 Identifying key virulence factors in oomycete pathogens

The findings presented in Chapter 4 demonstrate a comprehensive view of some of the major sub-proteomes of *P. cinnamomi.* The data obtained from this research concluded that the mycelia are geared towards saprophytic growth, trying to maintain sufficient growth within a plant host by breaking down host tissue and using it as a nutrient source. This coincides with findings in other *Phytophthora* species where their biochemistry focuses on metabolism, nutrient transport, and biosynthesis (Savidor et al., 2008; Hosseini et al., 2015). The biochemistry of zoospores amongst *Phytophthora* species has previously been elusive, not providing a complete system of how these cells function. The data obtained in Chapter 4 provided some interesting insight into the biochemical focus of the zoospores. It showed a significantly reduced metabolism, with a focus mainly on energy production to fuel the motor complex and response to external signals.

Our model provides a baseline for future research into the pathogenicity and virulence factors of *P. cinnamomi.* Evidence of necrosis induction factors was obtained by inoculating a model host with the purified extract which could potentially be effector proteins. This should be tested further using an additional protein purification step to confirm if these observations are caused by a protein and not other molecules such as metabolites. To our knowledge, this is a first report on the evidence of a proteinaceous

necrosis inducing factor in *P. cinnamomi*. If these candidates are further scrutinised and confirmed to be effectors, they can be exploited as tools the breeding of resistance genes in crops to trigger defence responses and improve the prospects of crop yield (Vleeshouwers and Oliver, 2014; Lenman et al., 2016; Chepsergon et al., 2021).

Although a protein profile of the mycelia and zoospores was obtained, delving deeper into these cell types could provide valuable insight into more potential key biochemical factors that could be exploited for disease control. For example, by further elucidating the motor complex and signalling network of the zoospores, core biological factors could be revealed and used as targets for chemical control resulting in the reduced spread of *Phytophthora.*

Virulent secreted proteins are characteristic traits amongst *Phytophthora* species (Kamoun, 2009). A shotgun proteomic approach provides the opportunity of identifying multiple virulence factors. The secreted proteome of *P. cinnamomi* revealed a plethora of virulence factors and caused a hypersensitive response on a model host. It is well known that secretome produced under *in vitro* conditions with no host material can results in the production of molecules that are associated with phytopathogenicity. While this is bioenergetically costly, this may prime the pathogen ahead of infection for when it encounters a suitable host (Tan and Oliver, 2017). Now that a set of virulence proteins have been identified, candidate effector pipelines can be applied to determine which of these proteins most likely facilitate infection in the host. This can be achieved through RNA interference which silences effector genes and determines if they confer virulence (Weiberg et al., 2013). Some methods such as transformation of gene deletion may be more difficult as *P. cinnamomi* has a diploid genome that contains two gene copies at the same locus (Engelbrecht et al., 2021). However, a system of transient expression of effectors into a host can be used to determine which candidates cause a necrotic response in a host (Dodds et al., 2009).

This study was restricted to the mycelia, zoospores and secreted proteins of *P. cinnamomi,* Other important sub-cells such as the oospores, chlamydospores, cysts, and appressoria could also provide important insight into the pathogenicity of *P. cinnamomi.* As these sub-cells are difficult to produce *in vitro,* their purification in sterile conditions would require optimisation. Nonetheless, obtaining a complete reference proteome profile of all life stages such as components of the cell wall could provide additional candidates as targets for chemical control (Theis and Stahl, 2004). Additionally, I did not perform proteome analysis on the zoospore secretome due to low protein yield that is insufficient for mass spectrometry analysis. I anticipate that the zoospore secretome will reveal virulence proteins such as candidate effectors that are required to establish cyst formation and penetration of the host tissue.

6.3 Advances in the discovery of the mode of action of phosphite and resistance

Phosphite is the only chemical used to combat dieback disease and the mode of action is not well understood. Both direct action on the pathogen and indirect through the plant system have been suggested in the literature however, the biochemical mechanisms in which it does this are not confirmed. The proteomic data presented in Chapter 5 has provided biochemical evidence on the direct and indirect mode of action of phosphite.

Growth inhibition and morphological changes have been observed in several oomycete species as a direct response to phosphite (King et al., 2010). However, the biochemical changes inflicted on the pathogen by phosphite have not been closely observed. The data presented in Chapter 5 demonstrates change in abundance of metabolic related proteins that alter the metabolic process in the sensitive isolate as a result of direct treatment of phosphite. I made particular note of the disruption of mitochondrial respiration, which is a common target in many fungicides (Li and Calderone, 2017). I provided two possible avenues of the mode of action of phosphite directly on the pathogen; that of mitochondrial respiration, and as a multi-site substrate.

These biochemical responses were not observed in the resistant isolate, which points to three possible mechanisms of resistance. If mitochondrial respiration is the target of phosphite, then the resistant isolate may have adapted the binding sites of the mitochondrial complexes (Fernández-Ortuño et al., 2008). In the case where phosphite is a multi-site oomyceticide, it is possible that the resistant isolate developed multi-site resistance, or more likely, adapted its transmembrane transporters to manage phosphite treatment. This could include blocking phosphite from entering the cell or efficiently remove phosphite from inside the cell. This poses a key question of whether phosphite can translocate into the cells of resistant isolates. If the genome of CPSM366 becomes available, the mass spectra from Chapter 5 could be used to provide a more accurate representation of the genome. Performing comparative transcriptomics and proteomics between these two genomes may lead to the discovery of polymorphisms that cause resistance.

I observed an increase in photosynthesis and metabolism-related proteins in the phosphite-treated lupin, along with an abundance of defence-related molecules. Metabolic responses to phosphite have been noted in the literature, along with increased expression of jasmonic and salicylic acid however there seems to be an oversight in the literature linking these responses together (Eshraghi et al., 2011; Machinandiarena et al., 2012). They have been viewed independently and as such, a model such as our *in planta* model has not yet been proposed. Interestingly, the modification of basal metabolism as a result of phosphite treatment hinders the growth of sensitive *P. cinnamomi* and acts as a biostimulant in lupin. This suggests that phosphite may interfere with or alter metabolism in a number of organisms and should be investigated across a wider spectrum of pathogens and hosts.

The photosynthetic response induced by phosphite may be reflective of a greening effect. The greening effect has been observed in fungicides where their mode of action not only kills fungal growth in plant systems but also alters plant metabolism and subsequently results in more crop/fruit production (Eiiner, 2005; Spitzer et al., 2012; Kyveryga et al., 2013; Tambascio et al., 2014). No physiological changes were observed

between the phosphite treated and untreated lupins however these were only 6-day-old seedlings. If the detected biochemical changes are a result of a greening effect, it would be useful to see the systemic changes throughout the plant, particularly in the leaves where photosynthesis primarily occurs. In addition, as these experiments were performed in a soil-free system, a long-term field trial of phosphite treatment would provide invaluable insight into whether these effects of phosphite can be replicated in the field. In addition, there could potentially be a prospect for the use of phosphite to improve the productivity of crop production irrespective of *P. cinnamomi* infestation.

Further experimentation could also be applied to the phosphite treated lupin that was subsequently infected with the two isolates of *P. cinnamomi.* Introducing this additional variable would be difficult to navigate in a proteomic experiment, looking at particular subsets of proteins within these treatments could inform on how the resistant isolate behaves compared to the sensitive isolate in a plant model. Similarly, we can simultaneously observe biochemical differences in the plant when treated with the two isolates that could lead to deeper understanding of the plant-oomycete feedback system.

6.4 Concluding remarks

This research has provided three foundational resources for *P. cinnamomi* biology; an improved genome, a proteome map, and a possible mode of action of phosphite, which lead to clear avenues for further research. With emerging resistance to phosphite, there is an urgent need to understand the biology of this resistance and move towards alternative methods of control.

The common aim of *Phytophthora* research is to discover biological features that can be exploited for control measures. This thesis aimed to gain an understanding of the pathogenicity of *P. cinnamomi.* The outcomes of this thesis have not only provided an in-depth biochemical snapshot of the pathogen, its virulence, and the effects of phosphite but revealed several key avenues of research to further pursue, which cannot have been identified without this research.

7. References

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Appendix 1- Attribution statement 1

To whom it may concern,

I, Christina Andronis, contributed the following to the publication entitled "Gene validation and remodelling using proteogenomics of *Phytophthora cinnamomi,* the causal agent of dieback" Frontiers in Microbiology. 2021; 12: 1519:

I performed the experimentation and data analysis except for:

- The scripts associated with mapping 6-frame peptides to their genomic location, which were provided by Dr James Hane

Technical advice was provided for experimentation by Dr Giles Hardy, Dr Scott Bringans and Dr Richard Lipscombe, and data analysis was performed with Dr James Hane.

I prepared the manuscript with Dr Kar-Chun Tan. Additional feedback and comments were provided by all other authors.

Christina Andronis

I, as co-author, endorse that this level of contribution by the candidate indicated as above is accurate:

Dr Kar-Chun Tan

Dr James K Hane

Dr Scott Bringans

Dr Giles E S J Hardy

Dr Silke Jacques

Dr Richard Lipscombe

Appendix 2- Attribution statement 2

To whom it may concern,

I, Christina Andronis, contributed the following to the publication entitled "Comparative sub-cellular proteome analyses reveals metabolic differentiation and production of effector-like molecules in the dieback phytopathogen *Phytophthora cinnamomi*", which has been submitted to the Journal of Proteomics with minor revisions submitted to the journal and now awaiting further comments:

I performed the experimentation and data analysis. Experimental design and proteomics work was performed with Dr Richard Lipscombe. I prepared the manuscript with Dr Kar-Chun Tan and Dr Silke Jacques.

Additional feedback and comments were provided by all authors.

Kar-Chun Tan, Richard Lipscombe and Silke Jacques provided project supervision

Christina Andronis

I, as co-author, endorse that this level of contribution by the candidate indicated as above is accurate:

Dr Kar-Chun Tan

Dr Silke Jacques

Dr Richard Lipscombe

Appendix 3- Attribution statement 3

To whom it may concern,

I, Christina Andronis, contributed the following to the publication entitled "Proteomic analysis revealed that the oomyceticide phosphite exhibits multi-modal action in an oomycete pathosystem". This manuscript has been submitted to Pest Management Science.

I performed the experimentation and data analysis.

I prepared the manuscript with Dr Kar-Chun Tan, Dr Silke Jacques and Dr Francisco J. Lopez-Ruiz. Proteomics work was performed with guidance from Dr Richard Lipscombe. Additional feedback and comments were provided by all authors.

Kar-Chun Tan, Silke Jacques and Richard Lipscombe provided project supervision.

Christina Andronis

I, as co-author, endorse that this level of contribution by the candidate indicated as above is accurate:

Dr Kar-Chun Tan

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Dr Richard Lipscombe