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

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ABSTRACT

Phytopathogenic oomycetes constitute some of the most devastating plant pathogens and cause significant crop and horticultural yield and economic losses. The phytopathogen *Phytophthora cinnamomi* causes dieback disease in native vegetation and several crops. The most commonly used chemical 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 targets the pathogen, the host, or both. Resistance to phosphite is emerging in *P. cinnamomi* isolates and other oomycete phytopathogens. The mode of action of phosphite on phosphite-sensitive and resistant isolates of the pathogen and through a model host was investigated using label-free quantitative proteomics. *In vitro* treatment of sensitive *P. cinnamomi* isolates with phosphite hinders growth by interfering with metabolism, signalling and gene expression; traits that are not observed in the resistant isolate. When the model host *Lupinus angustifolius* was treated with phosphite, proteins associated with photosynthesis, carbon fixation and lipid metabolism in the host were enriched. Increased production of defence-related proteins was also observed in the plant. We hypothesise the multi-modal action of phosphite and present two models constructed using comparative proteomics that demonstrate mechanisms of pathogen and host responses to phosphite.

Significance: Phytophthora cinnamomi is a significant phytopathogenic oomycete that causes root rot (dieback) in a number of horticultural crops and a vast range of native vegetation. Historically, areas infected with phosphite have been treated with the oomyceticide phosphite despite its unknown mode of action. Additionally, overuse of phosphite has driven the emergence of phosphite-resistant isolates of the pathogen. We conducted a comparative proteomic study of a sensitive and resistant isolate of *P. cinnamomi* in response to treatment with phosphite, and the response of a model host, *Lupinus angustifolius,* to phosphite and its implications on infection. The present study has allowed for a deeper understanding of the bimodal action of phosphite, suggested potential biochemical factors contributing to chemical resistance in *P. cinnamomi*, and unveiled possible drivers of phosphite-induced host plant immunity to the pathogen.

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. *P. cinnamomi* is considered one of the top 10 most devastating oomycete pathogens due to its aggressive and resilient pathogenicity [1]. It has contributed to the decline of many native species, particularly woody plants, and overall biodiversity in native forests globally [2,3]. *P. cinnamomi* has caused significant economic losses from reduced crop yield in the agronomic industry. This necrotrophic pathogen attacks the roots of susceptible hosts and causes plant death *via* saprophytic growth [4,5]. The dynamic lifecycle of this organism drives its resilience and success as a plant pathogen [6]. Similarly to other *Phytophthora* species, its zoospores enable it to survive through harsh environmental conditions and thrive once conditions become more favourable [6]. This gives the pathogen an advantage over its susceptible hosts and therefore could pose a great risk

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in the context of climate change, where dieback disease has the potential to thrive in some environments depending on the environmental conditions [7–10]. Characteristically, oomycetes produce an assortment of virulence molecules throughout their life cycles which facilitate the infection process [11,12].

Phosphite is a commonly used commercially available chemical used to control *P. cinnamomi* [13]. Other fungicides such as fosetyl-aluminium, copper sulfate, copper chloride amongst others are effective and can be used in particular contexts of environment or crop type, however phosphite is still the standard in managing *P. cinnamomic* globally due to its efficiency of reducing disease load [14–17]. Inorganic phosphites are reduced forms of phosphate that are commonly used as oomyceticides to manage diseases caused by *Phytophthora.* This includes *P. cinnamomi, P. nicotianae, P. palmivora, P. capsici* and *P. infestans,* along with other oomycetes such as downy mildews (*Pseudoperonospora humuli* and *Bremia lactucae*) [18–22]. Phosphites are applied on native trees and horticultural crops by foliar spray or direct injection into the trunk where it circulates throughout the plant system [23]. 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 [23–25]. The only other strategies implemented to combat dieback disease are the use of tolerant rootstocks if they are available (such as commercially available avocado rootstocks) and hygiene measures applied to vehicles and personnel, which aim to minimise spread [26–29]. *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 [30,31]. 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 [19,20,32–34]. It has been reported that mycelial growth and sporulation are inhibited by phosphite *in vitro* [32,33]. *In planta*, the increase in transcription of defence-related genes associated with the salicylic and jasmonic acid pathways in *Arabidopsis thaliana* and potato crops has been demonstrated post-treatment with phosphite [19,20,34]. The effect of phosphite on potato leaves indicates an increase in defence responses and altered metabolism, glycolysis, and carbon fixation [34]. 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.

Resistance 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* [32,35]. Resistance to phosphite has also been reported in *P. ramorum, P. nicotianae, P. capsici, Bremia lactucae* and *Pseudoperonospora humuli* [21,22,36–39]. This is likely due to the prolonged use of phosphite and poses a significant threat to natural ecosystems and the agricultural industry. The prevalence of resistance is motivating the need for improved management strategies.

To elucidate the mode of action of phosphite on the pathogen and the host, we used a label-free quantitative proteomic approach. This study encompassed the effects of phosphite on a sensitive and a resistant isolate of *P. cinnamomi.* Additionally, we investigated the effects of phosphite on the physiology in a model plant system that is susceptible to dieback. *P. cinnamomi* is used as a model to understand phosphite resistance as it is treated with phosphite in the field and tolerant isolates have emerged. By using a shotgun proteomics approach, we obtained a snapshot of the biochemical processes that are altered as a result of treatment with phosphite and determined 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 and characterised by 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 [35,40]. To determine the level of phosphite sensitivity of both isolates, they were grown on solid Ribeiro's media amended with phosphite at the following concentrations - 0.0, 1.0, 2.5, 5.0, 10, 20, 40, 100, 500 and 1000 μg mL^{-1} [41]. Mycelial cultures were incubated at room temperature in the dark [42]. 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 [43].

These represented an untreated and a sub-lethal dose of phosphite causing a physiological effect and providing enough biomass for experimentation. Mycelia from both isolates were harvested for protein extraction by scraping from the entire surface of the plate (to obtain sufficient biomass), snap-frozen in liquid nitrogen and freeze-dried. The total 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

Lupinus angustifolius (cv. 'Tanjil', commonly known as the narrow leaf lupin) 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 three days at room temperature under natural light [44].

Three-day-old germinated seedlings were sprayed with a 0.5% pH 7 solution of phosphite (Sigma, St Louis, USA) using a hand-triggered spray bottle and untreated seedlings were sprayed with water [45–48]. This concentration was recommended by the Department of Agriculture Fisheries and Forestry of the Australian Government on application to lupin [49]. Seedlings were incubated on a light shelf (400 μmol m^{-2} s^{-1}) at room temperature with a 12-h photoperiod [44]. Lupin root tips were harvested one day post-treatment. 1.5 cm of root tips were excised and immediately snap frozen, freeze-dried, 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, each consisting of the root tips of three independent plants, were collected for each treatment.

To test the effect of phosphite on the colonisation ability of *P. cinnamomi,* the root tips of each seedling were inoculated with MU94–48 or CPSM366 24 h post-spray by placing a colonised five mm disc of Whatman paper grown-over with mycelia from an agar plate beneath the root tips [50]. Containers were placed back on the light shelf for three days and lesion scores from zero (no observable lesion) to three (dark brown necrotic root tips) were recorded. The lesion scores were compared using a *t*-test to determine if the observed differences were significant.

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 total ground mycelia and the samples were kept on ice for 30 min with regular gentle

mixing. The crude extract was centrifuged at 20,000*g* at 4 °C for 30 min, the solubilised proteins were decanted, and proteins were precipitated using six volumes of ice-cold acetone and incubated at −20 °C overnight [51].

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 min with regular gentle mixing. The crude extract was centrifuged at 20,000*g* at 4 °C for 30 min. 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 five minutes at 15,000*g* at 4 °C. The aqueous phase was removed and 500 μL of methanol was added. Samples were inverted and the supernatant was discarded. One mL of ice-cold acetone was added and the samples were incubated at −20 °C overnight [52].

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 of 200 mM methyl methanethiosulfonate. Samples were tryptically digested overnight at 37 °C at a ratio of 1:10, desalted on a Strata-X 33 um polymeric reverse phase column (Phenomenex, Torrance, CA, USA) and dried in a vacuum centrifuge [5,53]. The peptide concentration was estimated by a NanoDrop spectrophotometer (Thermofisher).

2.4. Mass spectrometry and data analysis

One μg of each sample was loaded on an Acclaim™ PepMap™ 100 C18 LC Column, two μm particle size x 150 mm (Thermo Scientific) and peptides were resolved with a gradient of 10–40% acetonitrile (0.1% formic acid) at 300 nL/min over 90 min 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 using the Sequest HT algrorithm for spectral matching and normalisation was applied using the normalisation spectral abundance factor [54]. Mass spectra from the *in vitro* assay were matched to the *P. cinnamomi* MU94–48 genome consisting of 26,151 protein-coding sequences [55]. 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 resistance. The mass spectra obtained from the *in planta* assay were matched to the *L. angustifolius* genome consisting of 39,339 protein-coding sequences [56]. For protein identification, one or more 95% confidence peptides were used. For label-free quantification, proteins with two or more 95% confidence peptides were used and for significant differential abundance, a *p*-value threshold of <0.05 was used. For relative quantification, the spectral count of identified peptides was used. Ratios and *p* values for quantitative analysis were generated using the default *t*-test hypothesis testing over biological replicates [54].

To elucidate the functions of the detected proteins and understand the biochemical differences between samples, Gene Ontology (GO), KEGG and Interpro were used [57–59]. These functional assignments of the proteome were obtained using Interpro Scan 86.0. 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 (Fig. 1). The minimum inhibitory concentration (MIC) for the sensitive and resistant 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.

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 (Fig. 2a). Of the common proteins, 171 were significantly higher in abundance in MU94–48 compared to CPSM699 and 90 were significantly lower in abundance (Fig. 2b). 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.

3.2.1. 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 (Fig. 3). In MU94–48, an enrichment of putative stress response proteins was observed including glutathione S-transferases (GST), thioredoxins, peptidases, proteasomes and

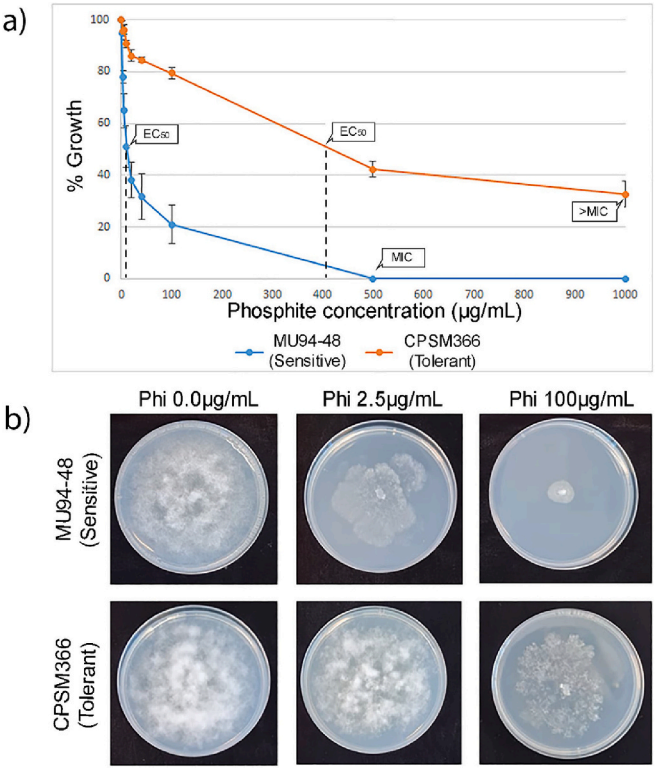

Fig. 1. *In vitro* growth inhibition of MU94–48 and a CPSM366 in response to phosphite treatment indicating the MIC and EC_{50} for each isolate. Images of mycelial growth on the phosphite-supplemented media are also displayed.

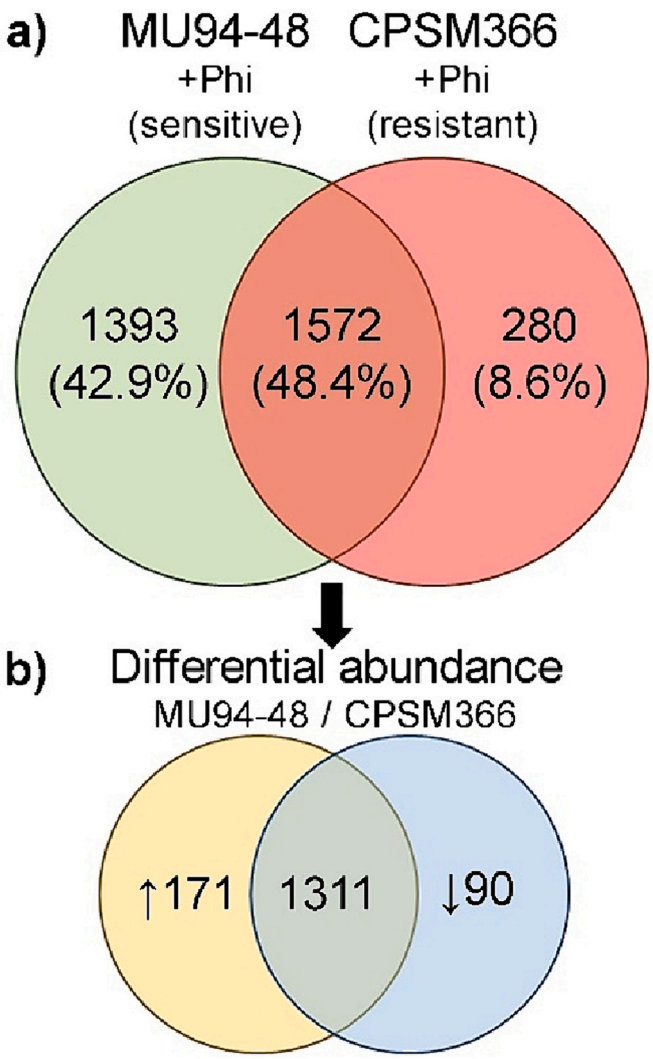

Fig. 2. Protein identification in phosphite-treated MU94–48 and CPSM366. a) Indicating the total number of unique and common proteins between the sensitive and resistant isolates. b) Differential abundance of the common proteins by label-free quantification shows the increased and decreased abundance 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 is shown in Supplementary Table 1.

proteolytic enzymes [60]. These indicate that MU94–48 is under stress when exposed to phosphite. Putative stress proteins were not observed to be differentially abundant in CPSM366 when treated with phosphite.

3.2.2. An increase in signalling in response to phosphite treatment

Enrichment of GOs in MU94–48 that are associated with protein signalling including phosphorylation (eg. ATPases, GTPases, PKs), and ubiquitination were observed [61] (Fig. 3b). 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. Proteins associated with inositol signalling and phosphorylation were significantly less abundant in MU94–48 treated with phosphite compared to CPSM366 (Fig. 3d). This is in contrast to CPSM366 where enrichment of proteins associated with the phosphatidylinositol binding GO was observed. Furthermore, proteins that are associated with regulating growth, development and metabolism such as TORC1 signalling and Wnt transmembrane signalling GOs were enriched when MU94–48 were treated with phosphite.

3.2.3. More transporters in MU94–48 than CPSM366 when treated with phosphite

An increased abundance and diversity of putative transporters were observed in MU94–48 treated with phosphite. This includes transmembrane transporters and intracellular transporters, along with transport facilitators such as COPI vesicle coatomer proteins, COP9 signalosome, armadillo-like proteins and clathrin proteins (Fig. 3d). This suggests significantly more removal of xenobiotics in MU94–48 than CPSM366.

3.2.4. Phosphite alters mitochondrial respiration in MU94–48

A significantly lower abundance of mitochondrial-associated ontologies was observed in MU94–48 when treated with phosphite. This was indicated by the over representation of the mitochondrial respiratory chain, cytochrome C oxidase, mitochondrial electron transport and mitochondrial ribosomal subunit GOs in CPSM366. This could be a result of oxidative stress in MU94–48 or may suggest that similarly to other fungicides, phosphite targets mitochondrial respiration [62,63]. KEGG ontology analyses of phosphite-treated MU94–48 and CPSM366 did not show changes in distinct metabolic pathway clusters but rather demonstrated evidence of metabolic disorder in MU94–48 (Supplementary Material 2). 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 artefacts of the differences between the isolates. It was observed that the proteome profiles of untreated and phosphite-treated MU94–48 and CPSM366 are comparable to those found between MU94–48 (Supplementary Material 3). This includes the enrichment of 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 fewer biochemical responses than when MU94–48 was compared to CPSM366. The full qualitative and quantitative dataset along with the associated GOs, KEGG orthologues and gene functions for all *in vitro* qualitative and quantitative comparisons are shown in Supplementary Table 4a-i.

3.3. Differential abundance of proteins between untreated and phosphite-treated Lupinus angustifolius

We then examine whether phosphite ellicit a physiological response in a susceptible host plant priming plant immunity to prevent infection by *P. cinnamomi.* To investigate this, the proteome of phosphite-treated and untreated L. *angustifolius* were compared. 498 proteins were unique in the untreated L. *angustifolius* sample and 245 were unique in the phosphite-treated Lupin (Fig. 4). 1725 proteins were common between the untreated and treated lupin samples of which 46 were significantly higher in abundance when phosphite was applied and four were significantly lower.

3.3.1. Phosphite increased the abundance of photosynthetic and carbon fixation proteins in L. angustifolius

GO enrichment and KEGG pathway enrichment analyses were used to examine the biochemical effects of phosphite in treated lupin (Figs. 5 and 6). It was observed that GOs containing proteins associated with photosynthesis and starch metabolism such as photosynthesis, photosystem units, geranylgeranyl reductase, chlorophyll biosynthesis, carbon fixation and TCA cycle were significantly enriched in phosphite-treated lupins. KEGG ontology clusters only found in the phosphite-treated lupin mapped to photorespiration, photosynthesis, reductive pentose phosphate cycle, isoprenoid biosynthesis and carbon fixation (Fig. 6). As lupin seedlings were grown under artificial light with roots

Fig. 3. GO enrichment and differential abundance between MU94–48 and CPSM366 treated with phosphite. a) and b) show GO enrichment in the phosphite-treated CPSM366 and MU94–48 respectively, obtained from the qualitative presence/absence dataset. 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 sensitive isolate at $P < 0.001$. d) Differential abundance of proteins between the sensitive and resistant isolate obtained from the quantitative dataset. Ratios are generated by the peptide signal of each protein in MU94–48 compared to CPSM366. *P* values depict the significance of the differential abundance with a significance cutoff of <0.05.

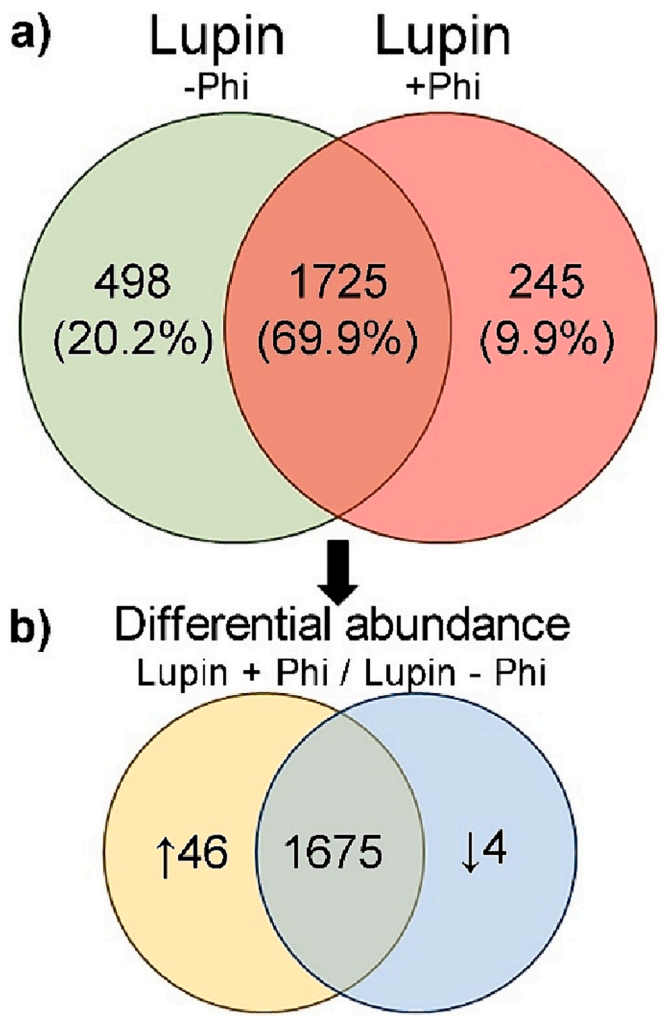

Fig. 4. Protein identification between untreated and phosphite-treated lupins. a) Indicating the total number of unique and common proteins between the untreated and phosphite-treated lupin. b) Differential abundance of the common proteins by label-free quantification shows the increased and decreased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

exposed leading to the development of photosynthetic-capable tissues, these were excised as part of the excised root tip for protein extraction (Fig. 7). The phenotype of the untreated lupins showed more lateral root hairs than the phosphite-treated lupins (Supplementary Material 5). Lupins were only monitored for six days during the course of the experiment and no other phenotypic differences were observed during this time.

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, 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 (Fig. 6). Phosphite treatment results in an increased abundance of proteins that are associated with photosynthesis, carbon metabolism and energy production in lupin.

3.3.2. 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-treated lupin samples (Fig. 5). This is also shown by the clustering of KEGG pathways in the phosphite-treated lupin including pyrimidine biosynthesis and keratan sulfate degradation (Fig. 6). Hence, an increase in gene expression and biosynthesis accompanies massive physiological and metabolic changes.

3.3.3. Phosphite triggers the accumulation of defence-related proteins in lupin

Phosphite has previously been reported to stimulate the production of defence-related molecules in plants relating to the salicylic (SA) and jasmonic acid (JA) signalling pathways [19,20,64]. 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 (Fig. 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.

Putative precursors to the SA pathway including isochorismatase and actin depolymerisation GOs were enriched in the phosphite-treated lupin. KEGG analysis revealed components associated with the JA pathway were only found in the phosphite-treated lupin. This shows the elevation of defence-related proteins including secondary metabolites were enriched and higher in abundance in the phosphite-treated lupin.

3.3.4. The effect of phosphite on MU94–48 and CPSM366 isolates during host infection

To test the effect of phosphite during host infection, *L. angustifolius* was treated with phosphite and subsequently inoculated with the sensitive or resistant isolate of *P. cinnamomi* (Fig. 7). Untreated lupin infected with MU94–48 and CPSM366 developed comparable lesions. When phosphite-treated L. *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 indicating resistance to phosphite *in planta.*

4. Discussion

The mode of action of phosphite in *Phytophthora* 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 other oomycetes 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* presents as an appropriate model for this study as it grows rapidly compared to native and horticultural *P. cinnamomi* hosts, it has a published genome sequence required for proteomics work and demonstrated high susceptibility to *P. cinnamomi* [56,65,66].

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} [32,67]. The EC_{50} MU94–48 falls within this range and can be considered highly sensitive to phosphite.

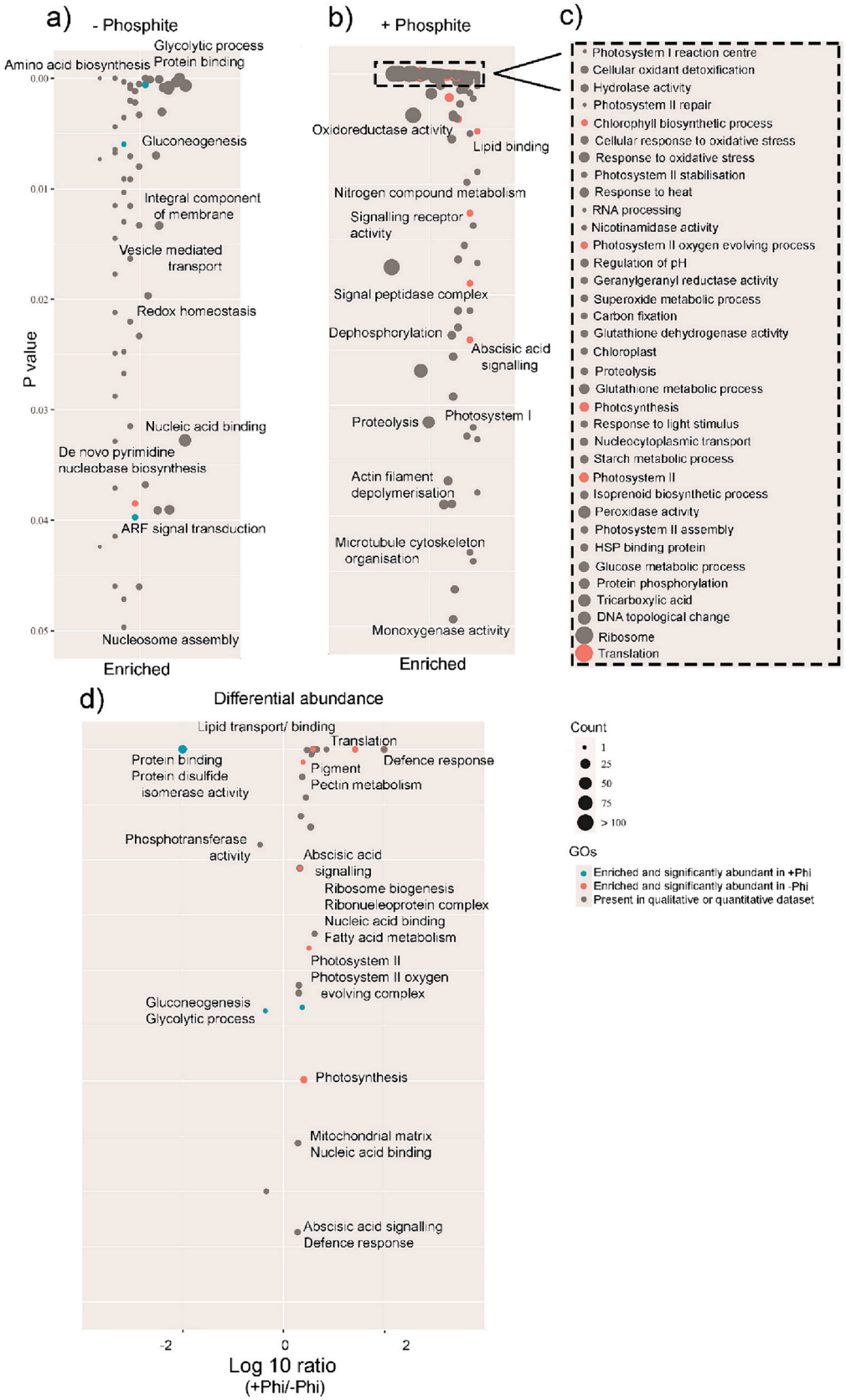

Fig. 5. GO enrichment and differential abundance between untreated and phosphite-treated lupin. a) and b) show GO enrichment in the untreated and phosphite-treated lupin respectively, obtained from the qualitative presence/absence dataset. The *P* value is generated by GO enrichment and each point is 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 obtained from the quantitative dataset. Ratios are generated by the peptide signal of treated lupin compared to the untreated control. *P* values depict the significance of the differential abundance.

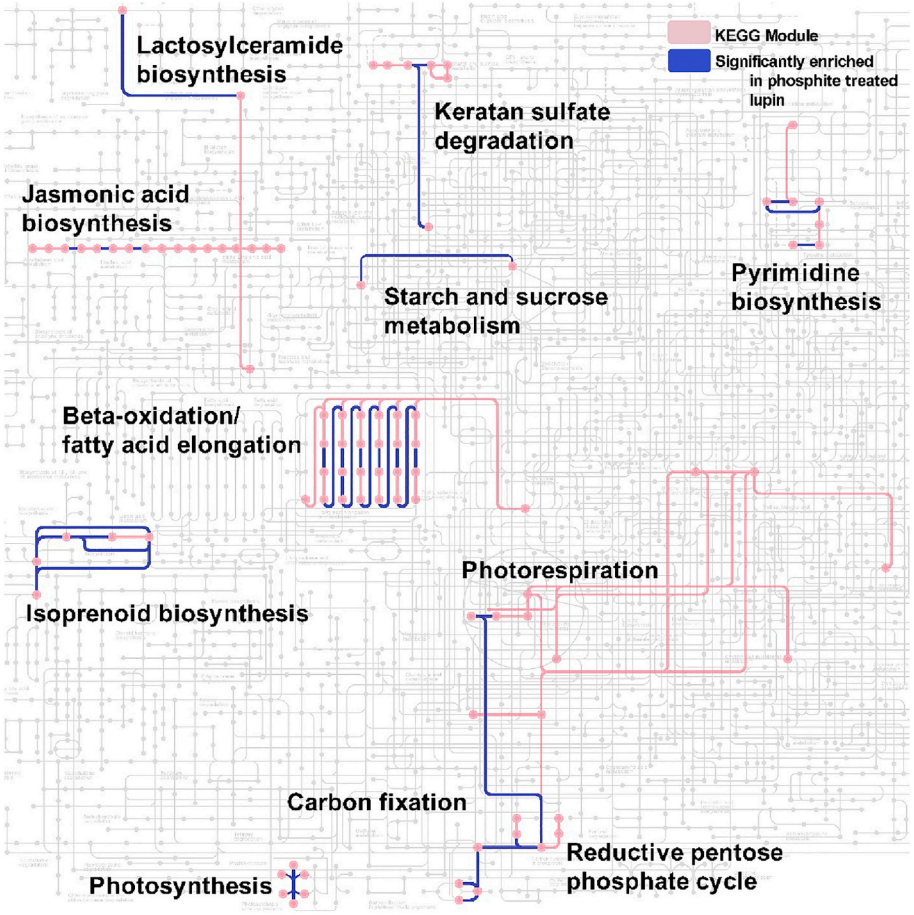

Fig. 6. KEGG analysis for biochemical pathways in response to phosphite treatment in lupin. Each dot represents a KEGG entry, and each line represents individual KEGG ontology identifiers. Pathway sets from overrepresented modules are labelled.

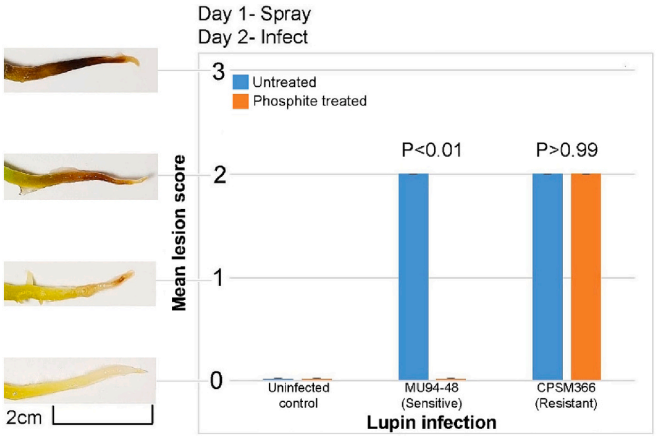

Fig. 7. Lesion scores of lupin roots when treated with phosphite and infected with the two isolates of *P. cinnamomi*. Lesion scores were taken on day 3. *P* values indicate significance between the untreated and phosphite-treated lesion scores. The full qualitative and quantitative dataset along with the associated GOs, KEGG orthologues and gene functions for all *in planta* qualitative and quantitative comparisons are shown in Supplementary Table 6a-c.

Reported resistant 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 by 18% [32,42,67,68]. *P. citrophthora* and *P. syringae* isolates 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 [36]. CPSM366 is therefore considered highly resistant to phosphite, highlighting the need to understand 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 significant growth reduction. The data obtained from this work was used to build a model suggesting the possible mechanisms by which phosphite directly affects the pathogen (Fig. 8). In the presence of phosphite, an abundance of putative stress proteins is observed in the sensitive isolate (Fig. 8a). We hypothesise that the pathogen is attempting to find ways to cope with the influx of phosphite by expressing GSTs and thioredoxins for detoxification [69]. Proteolytic 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 [60,70]. Putative stress response proteins were only observed in MU94–48 indicating that CPSM366 does not undergo abiotic stress when treated with phosphite. 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 (Fig. 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 [71]. This suggests that the pathogen attempts to cope with disrupted cell growth and metabolism caused by phosphite. 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

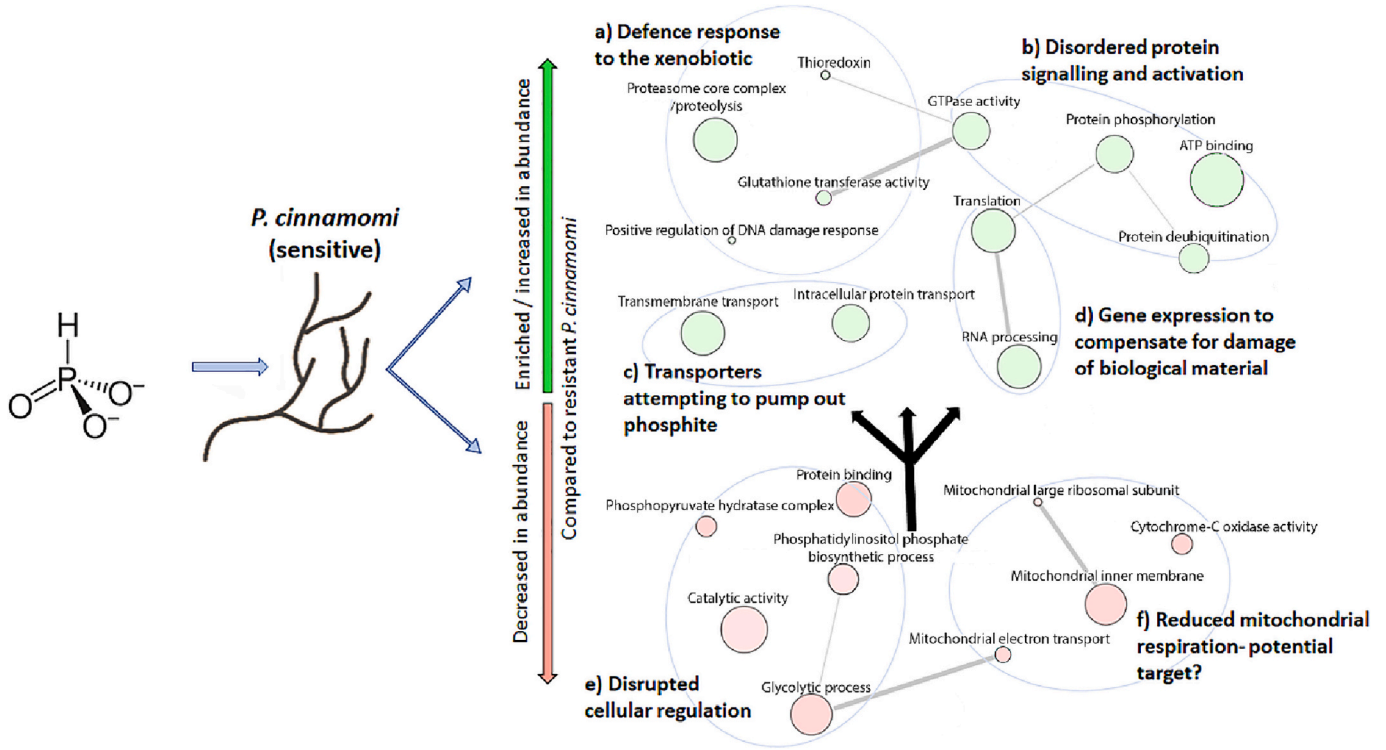

Fig. 8. A proposed model on the direct effect of phosphite on *P. cinnamomi* MU94–48 from the combination of qualitative and quantitative comparisons between MU94–48 and CPSM366. Green bubbles represent GOs enriched in MU94–49 and red bubbles represent GOs enriched in CPSM366. Black arrows indicate a possible cascading effect of core biochemical pathways that are disrupted as a result of phosphite treatment. The size of the bubbles represents the ratios of enriched GOs relative to each other.

gene expression to compensate for the loss of DNA, proteins and cell material through biosynthesis (Fig. 8d) [72]. Phosphite also alters canonical Wnt proteins which are core regulators of development. Inositol signalling and phosphorylation, which play important roles in aspects of cell growth, structure and signalling, were significantly lower in abundance in MU94–48 (Fig. 8e). These mechanisms of coping with extensive damage do not seem to be effective enough to protect the sensitive isolate from damage by phosphite.

A greater diversity of membrane transporters was observed in the proteome of MU94–48 than in CPSM366 (Fig. 8c). These transporters include ABC and MSF-type, which have been described to participate in the removal of xenobiotics from the cell [73,74]. Additionally, facilitators of intracellular transport were also enriched in MU94–48 suggesting that, unlike CPSM366, MU94–48 attempts to remove the phosphite or any toxic bioproducts that may be produced from phosphite metabolism. MU94–48 may be using transporters to pump out phosphite or toxic byproducts of phosphite metabolism to facilitate the high level of signalling. Similar findings have been shown in *Phytophthora ramorum,* where a phosphite-sensitive isolate was inoculated into phosphite-treated and untreated oak leaves. RNAseq analysis of the pathogen seven days post inoculation showed not only an increase in stress-related genes such as GST's but a range of transporters as a mechanism of efflux of toxins [75]. Alternatively, the abundance of transporters could be facilitating the influx of phosphite into the cell of the sensitive isolate. Our data shows that these are not abundant in the resistant isolate as these toxins may not be intracellularly accumulated or do not require removal.

Compared to the resistant isolate CPSM366, several constituents of the mitochondrial respiratory pathway were reduced in abundance in the sensitive isolate (Fig. 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 [76]. During these stress conditions, reactive oxygen species can disrupt components of the mitochondrial respiration chain causing cell death [77–79]. 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 [63,80]. For example, strobilurins are a broad-spectrum class of fungicides applied to control fungal crop diseases. Strobilurins bind cytochrome *b* complex III, inhibiting mitochondrial respiration [81]. Similarly, azoxystrobin blocks electron transport in mitochondrial respiration by blocking electron transport [81–83]. Evidence presented here might suggest phosphite exhibits a broader inhibitory activity as it seems to affect the pathogen's metabolism at multiple levels. Many fungicides such as chlorothalonil, folpet, thiram, sulphur and copper have multi-site modes of action, where several biochemical processes are disrupted [84,85].

The metabolic, signalling, regulatory and stress responses were not observed in CPSM366. If phosphite acts as a multi-site oomyceticide, we hypothesise that CPSM366 developed physiological adaptation to tolerate phosphite or alteration in target sites [86,87].

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 [19,20]. In addition, phosphite has been reported to act as a biostimulant in plants [88]. 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 (Fig. 9).

The biochemical differences between phosphite-treated and untreated L. *angustifolius* suggest an increase in the abundance of metabolic proteins that may have led to an elevation in general metabolism. This is evident from constituents of the photosynthetic process, carbon fixation and citric acid cycle suggest that phosphite is driving metabolism in plants (Fig. 9a). In addition, an increase in lipid and carbohydrate metabolism was observed, indicating that stored carbohydrates can be

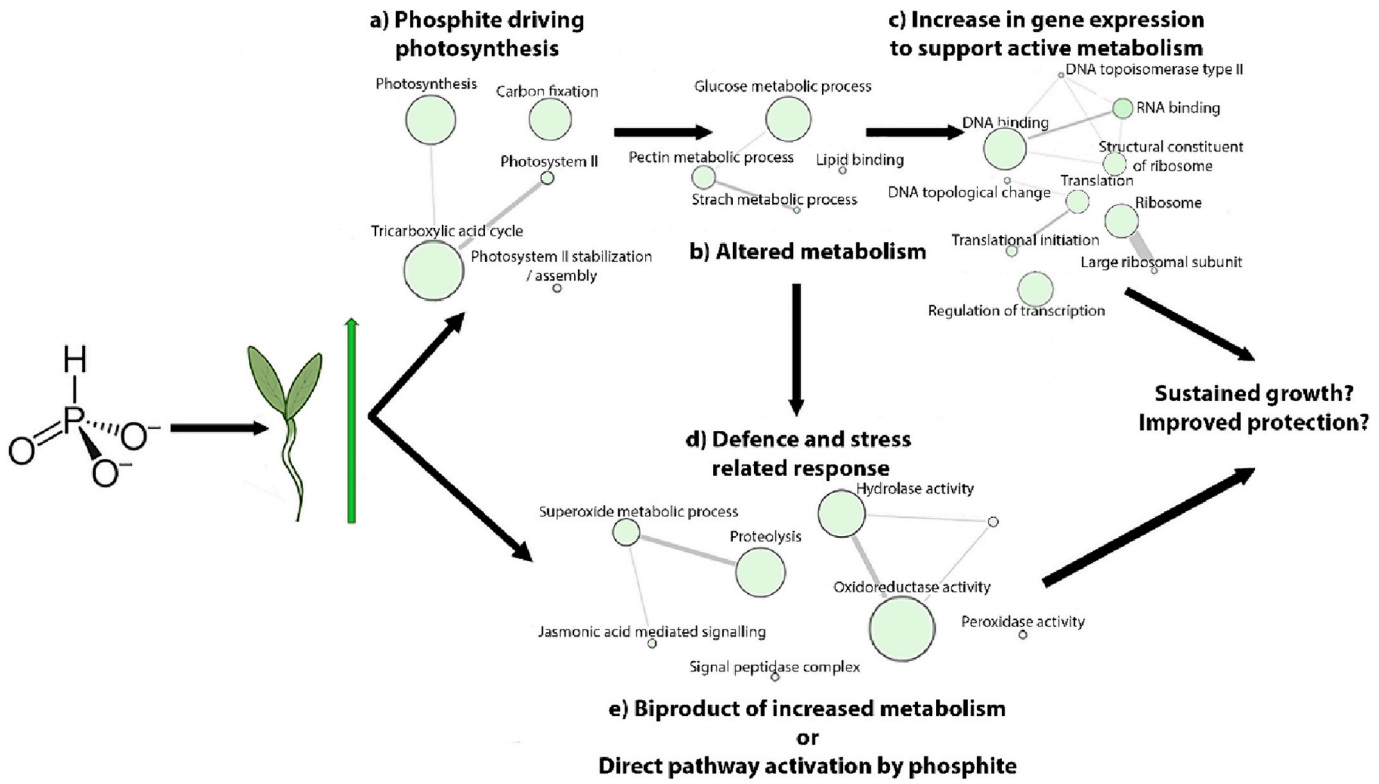

Fig. 9. A proposed model on the effects of phosphite on L. *angustifolius* from the combination of qualitative and quantitative proteome data between the untreated and treated lupin. Each green bubble represents a GO that is enriched in the phosphite treated plant. The size of the bubbles represents the ratios of enriched GOs relative to each other.

metabolised for energy generation (Fig. 9b). The reductive pentose phosphate cycle was observed in the KEGG ontologies of the phosphite-treated lupin and generates NADPH that feeds back into glycolysis [89]. Gluconeogenesis is significantly reduced in the phosphite-treated lupin highlighting that sugars are utilised for energy production in this state, not stored. This is reinforced by enrichment in gene expression to keep up with the metabolism and to cope with the increase in photosynthesis through the process of biosynthesis (Fig. 9c).

An increased metabolism has been observed in other phosphite-treated plants compared to untreated controls along with its bio-stimulant effect [34,88,90,91]. 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 [92,93]. In potatoes, phosphite application resulted in reduced seedling emergence time and increased leaf size and biomass [94]. This 'greening effect' has been demonstrated amongst other fungicide application in agricultural settings, where yield, biomass, leaf surface area, and protein content are increased as a result of fungicide application [95–97]. 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 [98–100]. Whether or not elevation in metabolism caused by phosphite translates to improved yield and growth in the host plant remains to be determined.

Defence-related gene expression has been demonstrated in several phosphite-treated crops. In potatoes, phosphite treatment caused a significant increase in the transcription of SA and JA [20]. Proteomic analysis of a similar system showed an increase in the abundance of peroxidases, glutathione S-transferase and proteinase inhibitors [101]. In the present study, enrichment of defence and stress-related proteins in L. *angustifolius* were detected as a result of phosphite treatment, which may prime 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 [102]. Superoxide metabolism and phospholipid binding composed of annexin genes were also enriched. Superoxide dismutase is used in plant defence against reactive oxygen species [103,104]. Isoprenoids identified in the phosphite-treated lupin are not only carriers in photosynthetic and respiratory electron transport but also have antioxidant and antifungal functions in plants and could potentially be synthesised in response to phosphite treatment in plants [105]. Defence-related ontologies with gene functions related to steroid chaperoning indicating programmed cell death and apoptosis were also abundant [106]. 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 SA [107–110]. The JA pathway is associated with a 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 [111,112].

In this system, phosphite is driving both metabolism and defence by directly promoting the respective biochemical pathways [20]. The production of stress molecules such as abscisic acid can at the same time be used as signalling molecules to trigger plant immunity [113]. The production of defence and stress-related molecules in the plant could also be acting in response to phosphite as a xenobiotic substance [114,115]. In the field, the ideal use of phosphite is part of a preventative strategy for the management of *P. cinnamomi* infection [23–25]. The *in planta* assay demonstrates that phosphite application does not reduce the observed lesion when lupin plants are infected with the resistant isolate. If the elevated defence response impacts the colonisation ability of *P. cinnamomi,* it is not observed in this system. Potentially

the induction of defence 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 modes of action of phosphite. This data demonstrates a comprehensive snapshot of the metabolic dysregulation in a sensitive *P. cinnamomi* isolate when treated with phosphite, suggesting it is a substrate for one or more core biochemical pathways. The resistant *P. cinnamomi* 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, particularly those relating to photosynthesis. As a bi-product of increased metabolism, more defence-related proteins are produced. We proposed probable mechanisms based on proteomic data that indicate a bi-modal mode of action of phosphite on both the pathogen and the host plant. Further studies are required to determine systemic changes in phosphite-treated L. *angustifolius* 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 presents opportunities for functional validation to determine the phosphite mode of action in crop protection.

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CRediT authorship contribution statement

Christina E. Andronis: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Silke Jacques:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Francisco J. Lopez-Ruiz:** Writing – review & editing, Visualization, Formal analysis. **Richard Lipscombe:** Writing – review & editing, Resources, Funding acquisition. **Kar-Chun Tan:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that the manuscript 'The oomyceticide phosphite exhibits multi-modal action in an oomycete pathosystem' was undertaken with no conflicts of interest. No additional financial support, affiliations, patents or copyrights are associated with this manuscript. Generative artificial intelligence has not been used in this manuscript.

Research data

Mass spectrometry data have been submitted to the Centre for Computational Mass Spectrometry under the MassIVE platform (doi:10.25345/C51834D2Q).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2024.105181.

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