Bioprotection - enhancing growth and health Research Programme

Bio-Protection
Bioprotection science for New Zealand

Plant defence responses to *Trichoderma* and elicitor treatments when challenged with *Diplodia sapinea* or *Phytophthora pluvialis*

Final report for tasks 4.6 and 4.7

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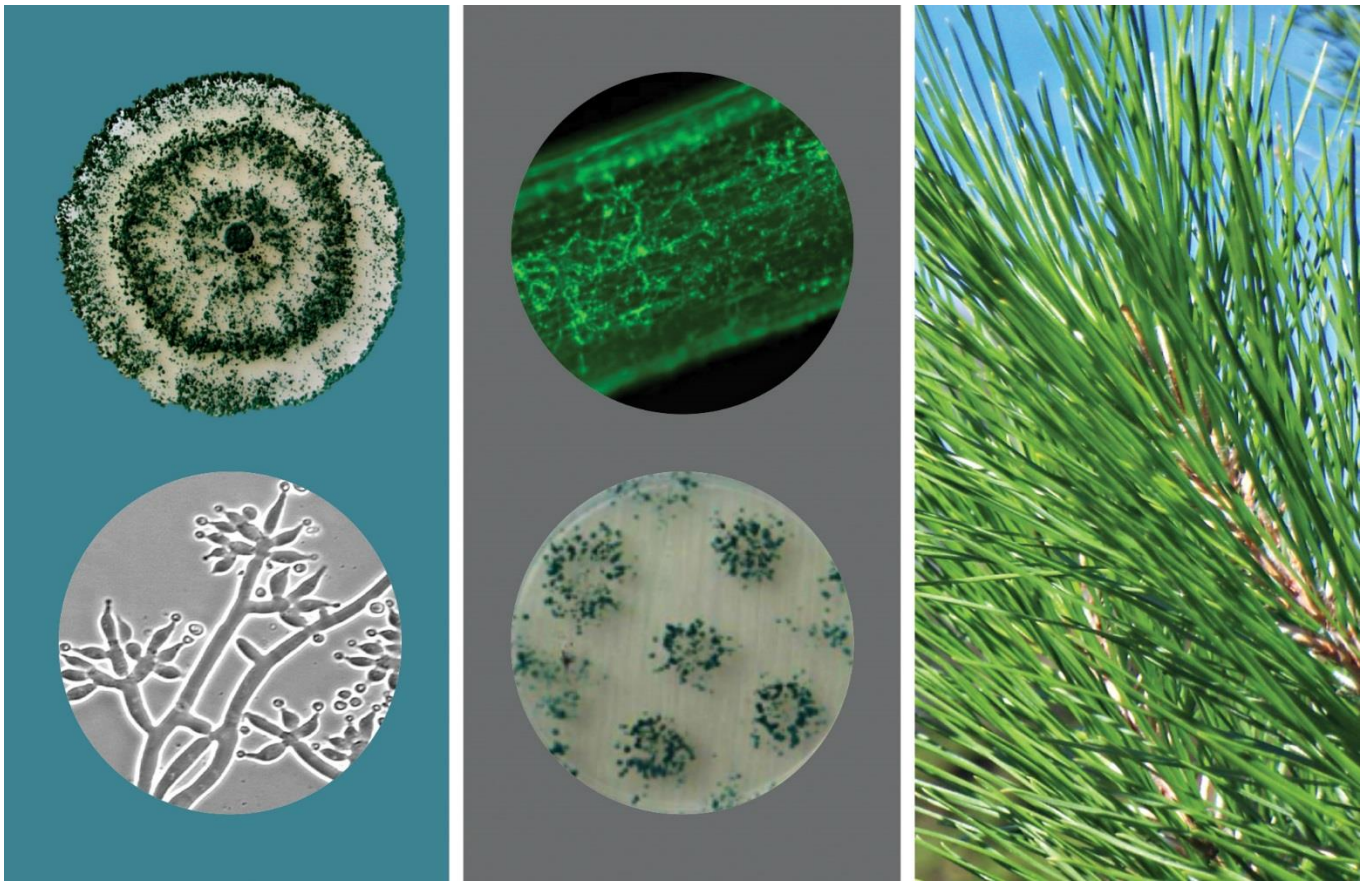

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Plant defence responses to *Trichoderma* and elicitor treatments when challenged with *Diplodia sapinea* or *Phytophthora pluvialis*

Rosie Bradshaw, Massey University; Tony Reglinski, Plant and Food Research; Beccy Ganley, Scion
February 2017

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EXECUTIVE SUMMARY

Report Title: Plant defence responses to *Trichoderma* and elicitor treatments when challenged with *Diplodia sapinea* or *Phytophthora pluvialis*
Authors: Rosie Bradshaw, Massey University; Tony Reglinski, Plant and Food Research; Beccy Ganley, Scion

The problem

The overall goal of the 'Bioprotection for foliar diseases and disorders of radiata pine programme' is to develop radiata pine with increased resistance to foliar diseases and disorders. The objective of this project was to determine if *Trichoderma* biological control agents or an elicitor (methyl jasmonate – MeJA) induced defence responses in pine needles when challenged with diplodia canker or red needle cast pathogens in artificially infected plants.

This project

In this project, *Pinus radiata* clones were pre-inoculated with *Trichoderma* spp. or MeJA treatments, and then infected with *Phytophthora pluvialis* (red needle cast) or *Diplodia sapinea* (diplodia canker). The plant materials used were Radiata Pine Breeding Company Elites that had been screened for resistance through the Healthy Trees, Healthy Future programme. Needle samples were taken at specific time points during the pathogen inoculation for transcriptomic and metabolomics analysis to determine changes in key compounds or genes during infection.

Key Results (tasks 4.6 and 4.7)

- A set of six defence-related *Pinus radiata* genes was selected from an initial pool of 33 genes for gene expression analysis.
- Expression of *Pinus radiata* defence-related genes showed clone-specific and pathogen-specific responses to challenge with either *P. pluvialis* or *D. sapinea*.
- With the exception of limonene and alpha pinene genes, defence-related gene expression levels were generally higher in plants challenged with *P. pluvialis* than with *D. sapinea*, and induction was more pronounced.
- The clone from the RNC 'resistant' seedlot did not appear to show higher levels of defence gene induction in response to *P. pluvialis* compared to the other clones.
- Defence-related gene expression levels were generally higher, or at similar levels, in MeJa- than in *Trichoderma*- treated plants. There was some evidence of Trichoderma-associated gene induction, but the patterns are complex and need further investigation.
- Terpenoid composition differed between the three clones. Of particular note was the 10-fold difference in limonene and β-phellandrene concentration across the three clones.
- Treatment with methyl jasmonate induced greater changes in terpenoid composition than *Trichoderma*, however, there is evidence of a differential clonal response to *Trichoderma* and in some cases an additive effect of *Trichoderma*+MJ on terpenoid content.
- There was little evidence to suggest that inoculation with *Diplodia sapinea* affects any terpenoid composition in needles.

Implications of Results for Client

Different clones clearly show different responses to pathogen challenge, elicitor and *Trichoderma* treatments at the molecular and biochemical levels. However the overall defence response profiles (in terms of expression levels and gene induction) differed in response to the two different pathogens. This concurs with the finding that these two

pathogens have opposite responses to MeJA and Trichoderma treatments and highlights the recommendation from the June 2016 report that MeJA provides an interesting tool to determine the fundamental resistance/susceptibility responses of radiata pine germplasm.

Further Work

We recommend the MeJA portion of this trial is repeated with enough plants to allow statistical replication to further investigate the mechanisms that influence resistance and susceptibility in radiata pine. This work would be complementary to the HTHF programme. Gene expression profiling by high-throughput RNA-seq would allow a broader picture of defence responses than is possible by qPCR alone. A more detailed investigation of the differential response of clones to *Trichoderma* and the additive effect of *Trichoderma*+MJ on terpenoid content may also be of some interest. Elicitors that operate via different biochemical pathways to methyl jasmonate should also be considered in order to broaden our fundamental understanding of elicitor/endophyte interactions and their potential to affect defence biochemistry.

Introduction

The overall goal of the 'Bioprotection for foliar diseases and disorders of radiata pine programme' is to develop radiata pine with increased resistance to foliar diseases and disorders. The main focus is to induce systemic resistance against foliar diseases by using endophytes and elicitors. In this project, two pathogens were selected to test the effect of *Trichoderma* spp. (endophyte) and methyl jasmonate (MeJA; elicitor) treatments on radiata pine. The two pathogens selected were *Diploida sapinea* and *Phytophthora pluvialis*.

Diplodia sapinea is a wound pathogen that can infect the branches or stem of radiata pine. This pathogen has been studied routinely in the Bioprotection programme, in particular used as an early screening method to select for biological control agents (BCAs) and elicitors that show promise in reducing disease symptoms (Reglinski et al, 2012) . BCAs that show potential have undergone more intensive screening against *D. sapinea* and have also been selected for testing against other pathogens, such as *P. pluvialis* and *Dothistroma septosporum*, as well as been used for field trials. The majority of the BCAs tested have been *Trichoderma* spp. In addition to BCAs, elicitors have also been screened against *D. sapinea* and MeJA, a phytohormonal elicitor involved in plant defence and other stress response pathways, has been shown to significantly reduce disease symptoms.

The second pathogen chosen, *P. pluvialis,* is a relatively new pathogen to forestry and causes a foliar disease in radiata pine known as red needle cast (RNC) (Ganley et al. 2014). Since a reliable screening method was developed for *P. pluvialis* for on plant and detached needle assays, select BCAs, previously screened against *D. sapinea,* have been screened against *P. pluvialis*[1] [2]*.* Reductions in disease symptoms using these select BCAs have not been consistently observed. However, the same plant material has not been used between the *P. pluvialis* and *D. sapinea* screening assays, making it difficult to compare results between assays. Furthermore, the elicitor MeJA has never been tested against *P. pluvialis*.

To obtain a more comprehensive picture of induced systemic resistance against foliar diseases using Trichoderma and MeJA, the defence responses of radiata pine to these treatments, as well as to pathogen challenge, were investigated using molecular and biochemical assays. The molecular analyses involved studying expression of a sub-set of 33 candidate pine defence genes using a quantitative PCR assay. The biochemical analyses focused on monoterpenes and phenolics that have been shown to be elevated in response to MeJA in radiata pine seedlings (Gould et al 2009).

In this project industry relevant clones (Radiata Pine Breeding Company Elites) that were known to be resistant or susceptible to *P. pluvialis* were used. The material was pre-inoculated with the same *Trichoderma* spp. and MeJA treatments so direct comparisons could be made when the material was subsequently challenged with *P. pluvialis* and *D. sapinea*. Material from both experiments was harvested for gene expression and metabolite analyses. The main objectives of this project were to determine if:

1. *Trichoderma* spp. and MeJA treatments could reduce disease symptoms against *P. pluvialis* and *D. sapinea*, [June 2016 report]
2. The effect of the treatments against *D. sapinea* reflected the effects observed against *P. pluvialis*, [June 2016 report]

[1] Ganley R and M Bader. (2014).Task 3: *Testing biological control agent (BCA) inoculated material against* Phytophthora pluvialis *using a detached needle assay.* Scion internal report (SIDNEY output 53018).

[2] Ganley R and M Bader. (2015). Task 1.4 *Testing biological control agent (BCA) inoculated material against* Phytophthora pluvialis in planta. Scion internal report (SIDNEY output 56160).

3. Pre-inoculated *Trichoderma* spp. could still be detected at the end of the experiment, [June 2016 report]
4. *Trichoderma* and/or MeJA treatments were associated with altered expression of pine defence genes, [This report]

This is the first time in this Bioprotection programme that a comparative assay between different pathogens, using the same host plant material and treatments has been trialled.

Materials and Methods

Plant material

Cuttings from 3 clonal *Pinus radiata* (radiata pine) lines were used for this experiment (Table 1). The cuttings were from Radiata Pine Breeding Company material that had been screened for resistance to red needle cast in the Healthy Trees, Healthy Future (HTHF) programme.

Table 1. Clone number and predicted disease resistance against red needle cast and dothistroma needle blight of radiata pine material.

Clone	RNC Resistance (HTHF rank)	Estimated dothistroma BV	Number of plants
Clone 1	Resistant (HTHF = r5)	27.7	112
Clone 2	Susceptible (HTHF = s9)	19.8	84
Clone 3	Susceptible (HTHF = s5)	28.4	112

All radiata pine material was propagated and maintained at Scion.

Treatments and experimental design

Two controlled environment assays were undertaken testing biological control agent (BCA) and elicitor treatments against two different pathogens *D. sapinea* (diplodia canker) and *P. pluvialis* (red needle cast). Radiata host material and preparation; BCA and elicitor treatments; and assay design were the same, where possible, between both experiments to allow comparison of results both within and across diseases.

A control and three treatments were tested:

1. Control – water only
2. Trichoderma
3. Trichoderma and MeJA combined
4. MeJA

Trichoderma inoculations were made 7 months prior, and MeJa treatments 14 days prior, to challenge with the *P. pluvialis* or *D. sapinea* pathogens. Please see June 2016 report for full details of methods.

Needle and root sampling

Needle material was sampled from both the *P. pluvialis* and the *D. sapinea* trials for gene expression and metabolite analysis. Root material was also sampled at the end of the experiment to test for persistence of the *Trichoderma* isolates used.

In the *P. pluvialis* inoculation four needles per clone, per treatment were sampled at 0, 24 and 168 (7 days) hours post inoculation. Two of the needles were frozen in liquid nitrogen and stored at -80 ^{o}C and sent to Plant and Food Research for metabolite analysis. The remaining two needles were cut into approximately 0.5 cm lengths then submerged in 0.7

ml of RNAlater stabilisation solution (Sigma-Aldrich, St. Louis, MO, USA), maintained at room temperature overnight and then stored at 4 ^{o}C. These needles were sent to Massey University for gene expression analysis.

For the *D. sapinea* inoculation four needles per clone, per treatment were also sampled but at 0, 48 and 168 (7 days) hours post inoculation. Again, two of the needles were frozen in liquid nitrogen and stored at -80 ^{o}C for metabolites analysis. The remaining two needles were cut into approximately 0.5 cm lengths then submerged in 0.7 ml of RNAlater stabilisation solution (Sigma-Aldrich), maintained at room temperature overnight and then stored at 4 ^{o}C. These needles were sent to Massey University for gene expression analysis.

Defence Gene Expression

For RNA extraction from needle samples stored in RNAlater solution, the needle tissue was surfaced-dried on absorbent paper, ground in a sterile mortar and pestle and RNA extracted from approximately 50 ug (fresh weight) of the ground tissue using a Spectrum™ plant total RNA kit (Sigma-Aldrich). The RNA suspension was DNAse treated using TURBO™ DNase (Life Technologies, Carlsbad, CA, USA). Then ~150 ng total RNA were used for synthesis of complementary DNA (cDNA) using a qSCRIPT cDNA Super mix (Quanta Biosciences, Gaithersburg, MD, USA) or a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Verification that the genomic DNA had been degraded by DNase treatment was determined by PCR amplification of actin gene sequence using primers ActinF and ActinR that bind either side of an intron, resulting in PCR products of 554 bp and 421 bp for gDNA and cDNA respectively. The PCR products were run on a 2% agarose gel with a 1 kb plus size marker ladder (Invitrogen, Carlsbad, CA, USA) as a control.

Relative quantitative real-time PCR was used to determine the expression levels of *Pinus radiata* genes using primers developed by Lisa Stanbra at Scion (Table 1 and Appendix Table A1). Two uL of the cDNA reaction was used as a PCR template, along with 5 uL of SensiFAST SYBR® No-ROX qPCR reagents (Bioline Reagents Ltd., London, UK). To determine which housekeeping genes were best for normalisation controls, four genes were tested (Actin, Tef1α, Gapdh, and RuBisCO) with ten samples. Using the geNorm algorithm developed by Vandesompele et al. (2002), the Actin and Tef1α genes were shown to have the most consistent levels of expression across all samples, so the mean expression values for these two genes were used for normalisation. These and the target genes all showed amplification efficiency values close to the expected 2.0).

For analysis of samples 2 uL of cDNA was PCR-amplified using SensiFAST SYBR® No-ROX qPCR reagents (Bioline) with a LightCycler® 480 II instrument (Roche Diagnostics, Ltd., Risch-Rotkreuz, Switzerland) and subjected to 45 cycles of PCR (98ºC for 10 sec then 15 sec at 60ºC and 20 sec at 72ºC) with an acquisition temperature of 72ºC. Crossing points (Cq) were used to determine the relative concentrations of the target gene expressed relative to the geometric mean of Actin and Tef1α gene concentrations.

The expression levels of 33 putative defence-related genes of *P. radiata* were screened by qPCR with 10 samples from clone 3 (Nil 0h, 24h, 168h; MJ 0h, 24h, 168h; Tri 168h; Tri+MJ 168h; nil 168h no RNC; Tri 168h no RNC)[Scion samples]. A second-stage screen involved 14 of the genes with 10 different samples (Tri+MJ 0h, 24h clones 1, 2 & 3; Tri 24h, 168h clones 2 & 3)[Scion samples]. Pre-screening involved one biological replicate. Subsequently, six genes were selected for analysis with the entire sample sets from Scion and Plant & Food Research (PFR). Three biological replicates will be analysed for all samples, but only one replicate (Scion sample) has been completed for all samples so far. Please see FOA July 2016 project report for details of how these samples were obtained. Primers for the six selected genes and the two normalisation control genes are shown in bold font in Table A1.

Needle Chemistry

Frozen needle samples were ground in liquid nitrogen and then stored at -80°C until analysis. Terpenoid extraction was performed using 3:1 n-hexane:diethyl ether containing 0.1 mg.mL-1 anethole as an internal standard as described by Gould et al., (2009). The target analytes were analysed by gas-chromatography mass spectrometry (GCMS) using an Agilent 6890 gas chromatograph coupled to an Agilent 5975 MSD. The injector was held at 250°C and 1 µL of sample extract and calibration standards were injected by autosampler using split injection and a split ratio of 1:20. Chromatographic separation was achieved using an Agilent DB5-MS glass capillary column (30 m x 0.250 mm i.d. x 0.25 µm film thickness). Helium carrier gas was maintained at a flow rate of 1 mL.min-1. The GC oven was programmed at 50oC (1 min), increased to 120°C at 4°C min-1, followed by 50oC min-1 to 200°C (5.0 min hold). The GCMS interface was held at 280°C and the mass spectrometer source and quadrupole temperatures at 230°C and 150°C respectively. Total ion spectra (50 to 300 m/z) and single ion monitoring (SIM) data were obtained simultaneously in synchronous scan and single ion monitoring mode. Calibration standards for each compound, ranging from 10 - 1000 µg.mL-1, were analysed together with the sample extracts. The target analytes were identified by comparison of retention times against certified standards and comparison of total ion mass spectra against the NIST mass spectral database. The concentration of each target monoterpene was determined from the compound specific mass ion responses obtained from SIM and comparing the relative responses of the monitored compound specific mass ions against those obtained from pure compound standards. Quantification of target analytes was completed by internal standard quantitation, with anethole as the internal standard, and analysis was performed using Agilent MSD Enhanced Chemstation software. All chemicals and reagents were obtained from Sigma-Aldrich unless otherwise stated.

Results and Discussion

Defence Gene Expression screen 1:
33 putative defence-related genes and one *P. radiata* clone.

It was not feasible to test the expression of all 33 defence gene candidates with the large number of samples (308) available, thus a step-wise approach was taken to determine the best candidate genes to use. Using *P. radiata* clone 3, three of the 33 primer sets tested (class IV chitinase, MAP kinase, pinosylvin synthetase) gave no amplification at all with any of the samples, thus were discarded from further analysis. The remaining 30 gene candidates were assessed using the following criteria to decide which to shortlist (see Appendix Table A2):

- How good is the gene prediction? Sometimes the identity of the gene was not clear from database comparisons (BLAST analysis). (column C)
- How specific is the amplification? Non-specific amplification was determined by melting curve analysis after qPCR. For example peroxidase 2 had very high levels of expression but double peaks indicating more than one gene was being amplified. (column D)
- Is the expression level moderate or high (relative to controls)? (column E)

Expression ratios were calculated for the 30 expressed genes to indicate the effects on gene expression of Trichoderma treatment, methyl jasmonate treatment or pathogen challenge, (columns F to J). Although these were only based on one replicate, they indicated which genes had strong responses to treatments.

Defence Gene Expression screen 2:

14 putative defence-related genes and three *P. radiata* clones.

On the basis of expression screen 1, 14 genes were selected for screen 2 (column K of Appendix Table A2). Alpha and beta pinene and limonene were included in the second screen despite their low expression, because biochemical assays at Ruakura indicated different levels of these compounds between clones. Accordingly, screen 2 was designed to compare clones, as well as to look at differences in expression over time with Tricho or Tricho + MeJa treatments (columns M to R). As a result of expression screen 2, six genes were selected for full analysis (column S). Alpha pinene was chosen over beta pinene as the identity of the latter gene was in doubt (it matched both alpha and beta pinenes in BLAST database analyses). Box 1 summarises key features of the selected genes. It is intended that further genes will be selected for full analysis in future.

Box 1: Key features of the six selected genes.

Alpha pinene
Monoterpenes such as pinenes and limonenes are associated with induced defence responses in conifers (Zulak et al., 2009). Although not expressed at high levels in expression screening rounds of the current project, the predicted α-pinene gene was selected because pinene levels increased following treatment with the defence elicitor methyl jasmonate (Zulak et al., 2009, Gould et al., 2009; Reglinski et al., 2012).

Limonene
Like alpha pinene, limonene gene expression was not high in screening rounds 1 and 2, but was selected because of the strong effect of tree genotype on production of limonene in this project (Ganley et al., 2016).

CCoAOMT
Caffeoyl CoA 3-O- methyltransferase is involved in G-type lignin biosynthesis in conifers including *P. radiata* (Wagner et al 2011; Pascual et al., 2016). It also has broader applications with a central role in phenylpropanoid modification (Vogt 2010) and was one of the most highly expressed of the genes used in the screening rounds.

Endochitinase
A predicted pathogenesis-related 3 (PR-3) family protein with closest similarity to *Pinus contorta* class I chitinase. Chitinases are induced in response to methyl jasmonate and to pathogen attack in conifers (Davis et al., 2002; Veluthakkal and Dasgupta 2010). Expression was moderately high in the expression screening rounds in this project, and clear induction was seen in response to both methyl jasmonate and *P. pluvialis*.

Phenylalanine hydroxylase
In *Pinus taeda* and other nonflowering plants, phenyalanine hydroxylase is located in chloroplasts where it catalyses the conversion of phenylalanine to tyrosine (Pascual et al., 2016; Pribat et al., 2010). Although its role in conifers is not known, it might regulate the flux of phenyalanine into the phenylpropanoid/lignin pathway. In the screening rounds expression appeared to be induced by methyl jasmonate but repressed by *P. pluvialis*.

Thaumatin-like protein (PR-5)
A predicted pathogenesis-related 5 (PR-5) family protein with antifungal activity. Expression of thaumatin-like PR-5 proteins is regulated by stress (biotic and abiotic) in poplars and pines (Petre et al., 2011, Veluthakkal and Dasgupta 2010). In screening rounds expression of this gene was induced by methyl jasmonate and there were clear differences in response between tree genotypes.

Defence Gene Expression:

Six genes and 3 clones: Effects of pathogen challenge with *P. pluvialis* or *D. sapinea.*

The effect of challenge with *P. pluvialis* or *D. sapinea* on expression of the core set of six genes at 168 h is shown in Figure 1. The results suggest that:

- There were different responses between clones for all six genes tested with both pathogens. For example clones 1 and 2 showed opposite responses of the thaumatin-like protein (PR-5) to *D. sapinea* challenge in the presence of *Trichoderma*.
- Four of the defence-related genes appeared to be induced in response to challenge with *P. pluvialis* (alpha pinene, thaumatin-like protein, endochitinase and CCoAOMT). However, when challenged with *D. sapinea,* only endochitinase amongst these four genes appeared to be induced; instead alpha pinene and CCoAOMT showed the opposite effect or no effect, whilst the thaumatin-like protein showed clone-specific differences as mentioned above.
- Clone 1 (RNC 'resistant') only showed higher levels of phenylalanine hydroxylase expression in response to *P. pluvialis* compared to the other more susceptible clones 2 & 3; all other genes tested appeared to show less induction in clone 1 than clones 2 and 3.
- Trichoderma treatments appeared to modulate gene expression in some cases, such as increasing limonene and alpha pinene expression in the PFR clone 2 samples, and phenylalanine hydroxylase induction in clone 1 (both pathogens).

In summary a comparison of gene expression in pathogen-challenged and unchallenged plants shows clone-specific and pathogen-specific responses. In general, clone 1 (from RNC 'resistant' seedlot) did not appear to show higher levels of defence gene induction in response to *P. pluvialis* compared to the other clones.

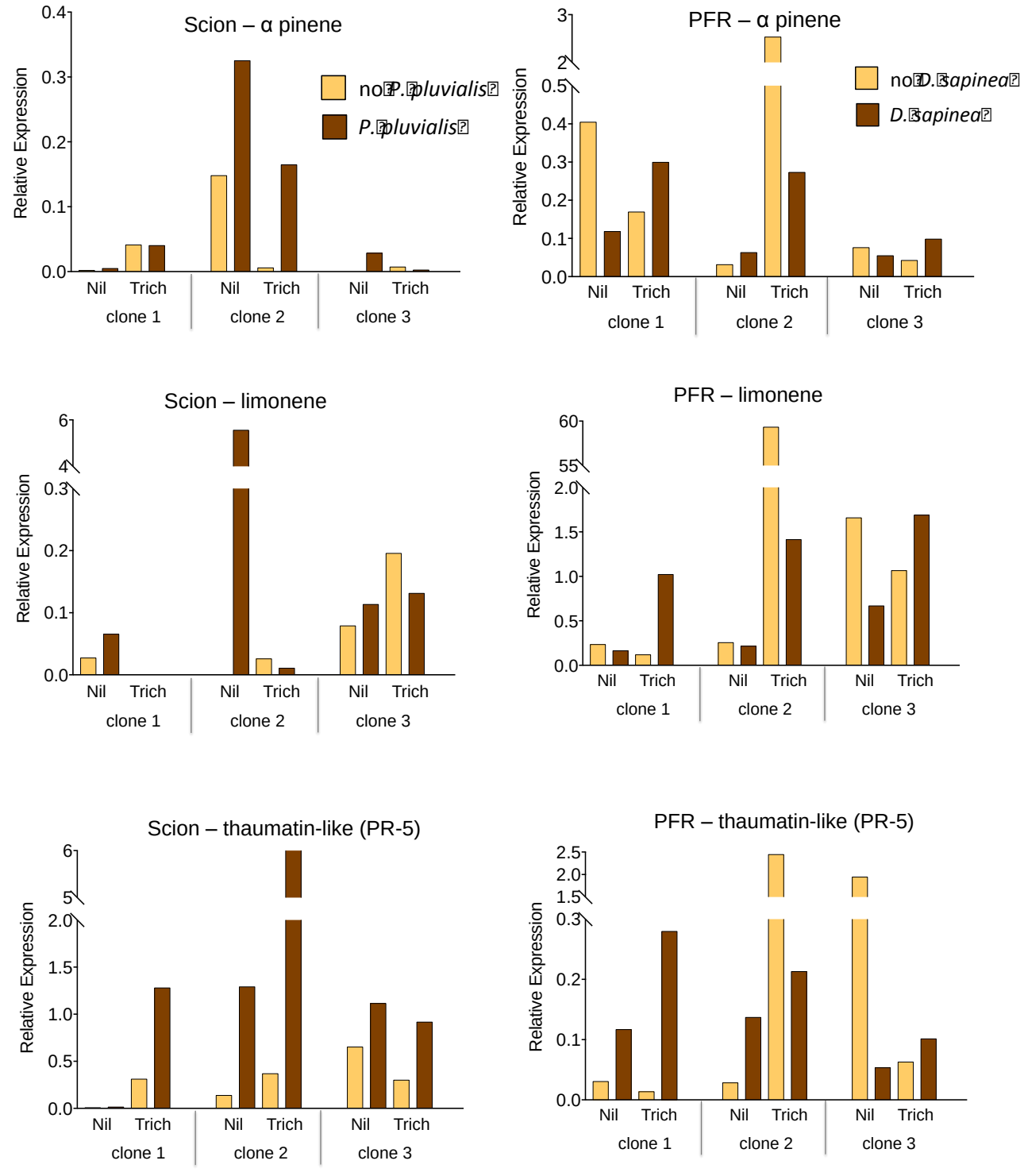
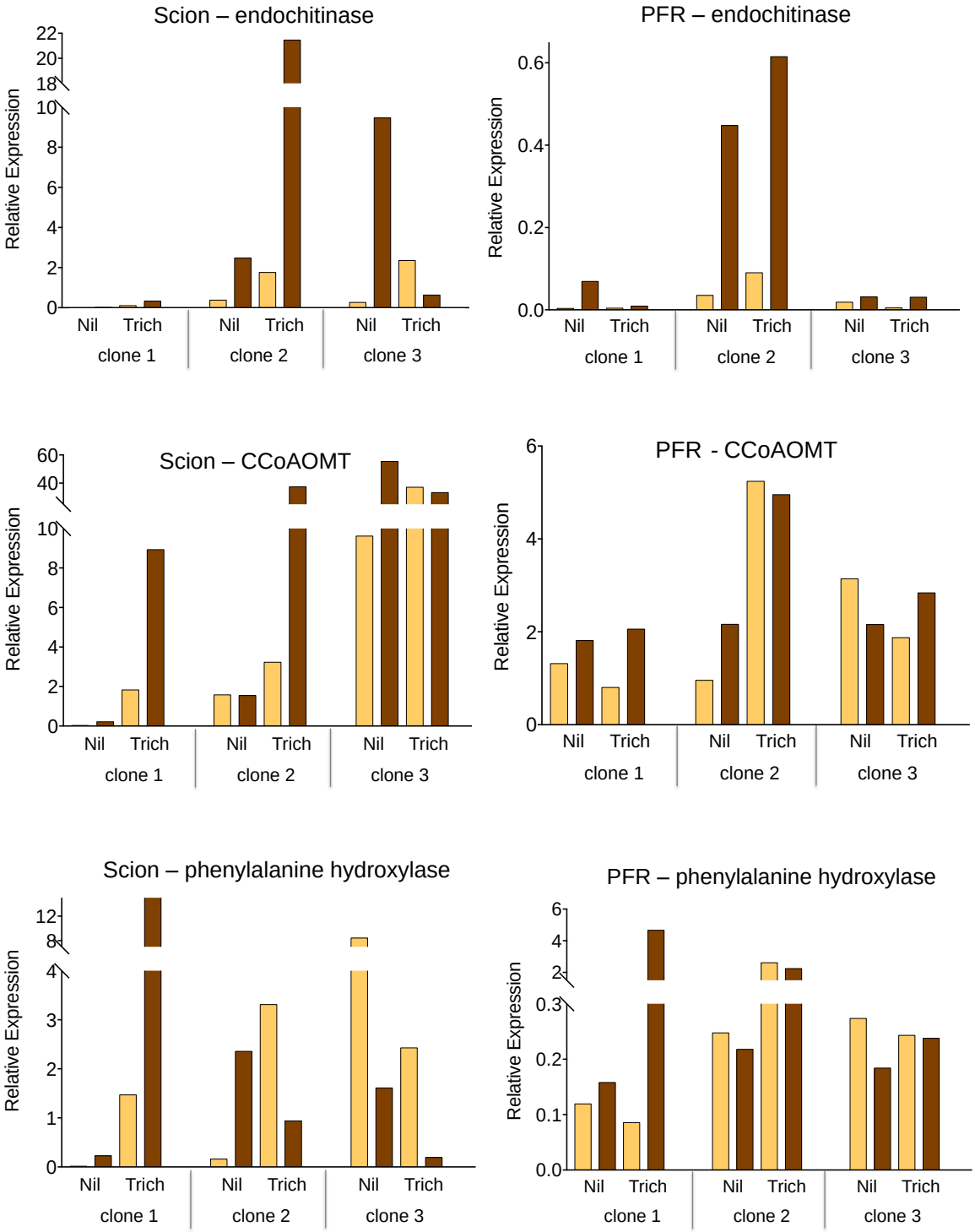


Figure 1. *Pinus radiata* gene expression responses to the presence of *P. pluvialis* (RNC pathogen) with and without *Trichoderma* treatment [Scion samples]. All samples were taken at 168 h (7 d) from trees treated with Trichoderma (Trich) or untreated (nil). Three independent *P. radiata* clones (1,2,3) were assessed. Brown bars indicate plants challenged with *P. pluvialis* (left) or *D. sapinea* (right); yellow bars are unchallenged controls.

Fig. 1 continued..

Scion samples (*P. pluvialis*) PFR samples (*D. sapinea*)



Defence Gene Expression:

Six genes and 3 clones: Effects of Trichoderma and methyl jasmonate treatments.

The effects on gene expression in response to Trichoderma and methyl jasmonate treatments over a time course are shown in Figure 2. All the trees were challenged with *P. pluvialis* (left) or *D. sapinea* (right) at time 0 h. The results suggest that:

- With the exception of limonene and alpha pinene, gene expression levels were generally higher in plants challenged with *P. pluvialis* than with *D. sapinea* (see Y axis numbers).
- In general, there was more induction of gene expression in response to challenge with *P. pluvialis* than with *D. sapinea*. This can be seen by the prevalence of blue (48 h, 168 h) bars in the Scion *P. pluvialis* samples (left) compared to the mostly grey bars (0 h) for PFR *D. sapinea* samples (right).
- There were different responses between tree clones for all six genes tested. For example, in response to *P. pluvialis* challenge, clone 3 treated with MeJa + Trichoderma showed the highest up-regulation of all genes tested except α-pinene and limonene.
- Gene expression levels were generally higher, or at similar levels, in MeJa- than in Trichoderma- treated plants. There are a few examples of possible Trichoderma-related induction of gene expression, but they are very clone- and pathogen-specific; for example in clone 2: endochitinase at 168 h (Scion) and CCoAOMT at 0 h (PFR). Further work is needed to investigate the effects of MeJa and Trichoderma.
- Lower alpha pinene gene expression levels in clone 3 (compared to clones 1 and 2) concur with the lower alpha pinene levels reported from biochemical assays in the FOA July 2016 report. Higher levels of limonene were also shown in biochemical assays with clone 3 (FOA July 2016 report); in contrast the limonene gene expression studies suggest lower, but more consistent, levels in clone 3.

Figure 2 (legend on next page):

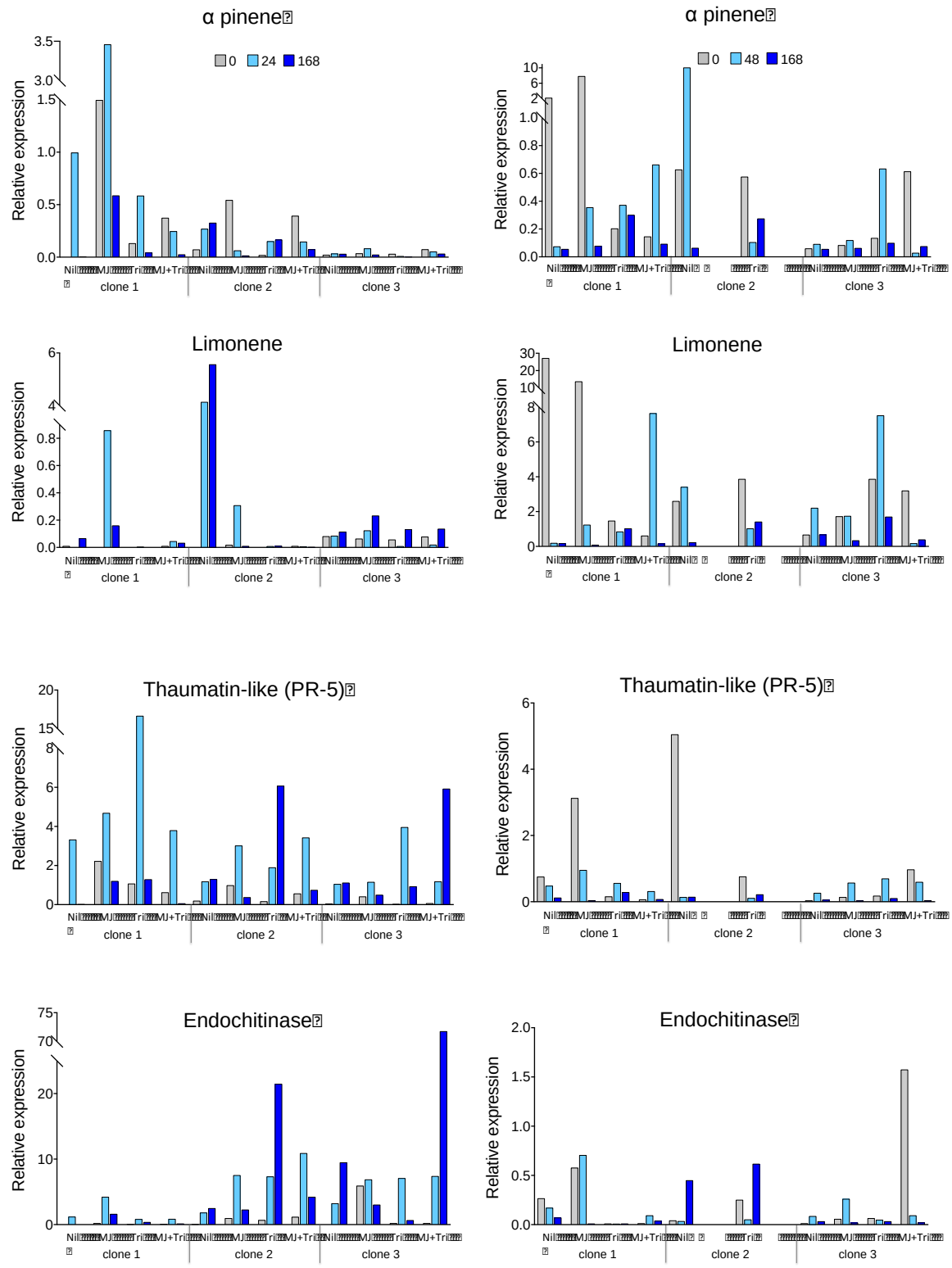


Fig. 2 continued.

Scion samples (*P. pluvialis*) PFR samples (*D. sapinea*)

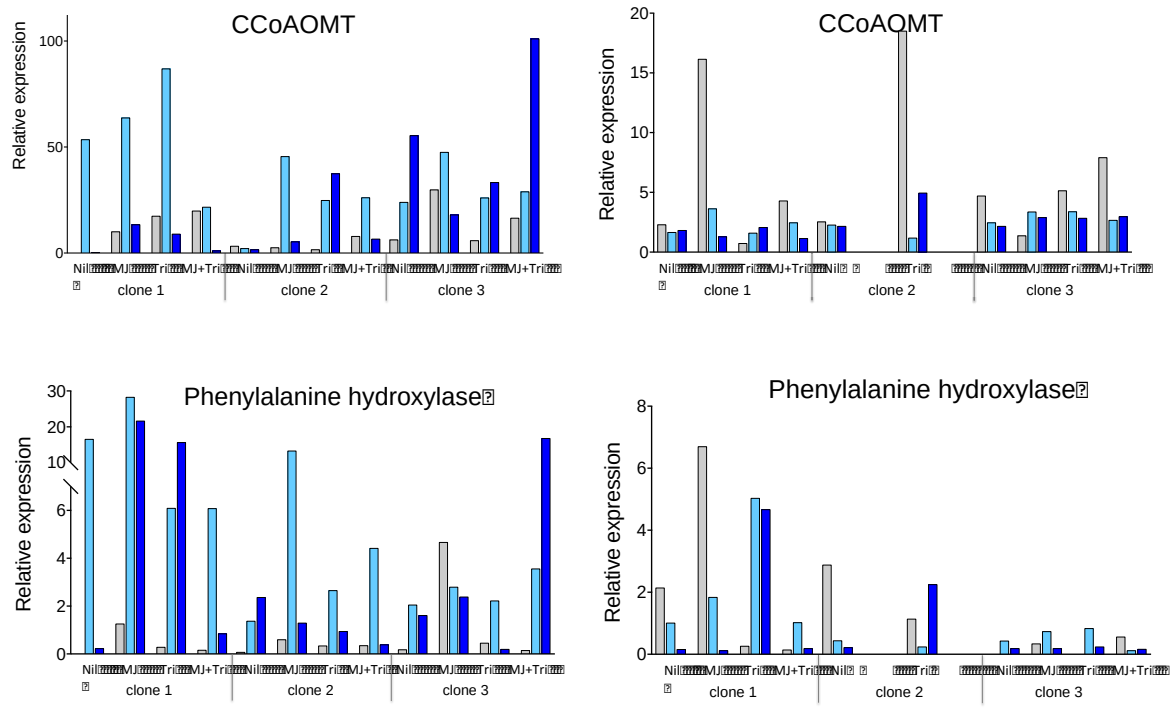


Figure 2. *Pinus radiata* gene expression responses to treatment with Trichoderma or methyl jasmonate (days after challenge with *P. pluvialis* [Scion] or *D. sapinea* [PFR]). Samples were taken at 0h (grey) 24/48 h (light blue) or 168 h since challenge with *P. pluvialis.* Expression of six genes was assessed in three independent *P. radiata* clones (1,2,3). Note that for clone 2 there were insufficient trees to include MJ or MJ + Tri treatments at PFR.

Needle Chemistry:

Ruakura experiment

The effects of treatment with MJ and *Trichoderma* and the subsequent response to challenge inoculation with D*. sapinea* on the concentrations of eight selected terpenoid compounds is shown in Figure 3 (the details of 10 compounds are available in Appendix Table A3).

Clonal effects - comparisons between the untreated controls:

- α-pinene concentration was 2x greater in clone 2 than in clones 1&3.
- Limonene concentration was over 10x greater in clone 2 than in clones 1&3.
- β-phellandrene concentration was over 10x greater in clone 1 than in clones 2&3.
- trans-caryophellene concentration was 2-3x greater in clone 1 than in clones 2&3.
- Inoculation with *D. sapinea* did not induce a change in terpene concentration in untreated plants over the duration of the experiment (168h).

Treatment effects - effects of MJ and *Trichoderma* (before inoculation with *D. sapinea*):

- MJ caused an increase in the concentrations of β-pinene, myrcene, & terpinolene in clone 1, and an increase in the concentrations of α-pinene, β-pinene, β-

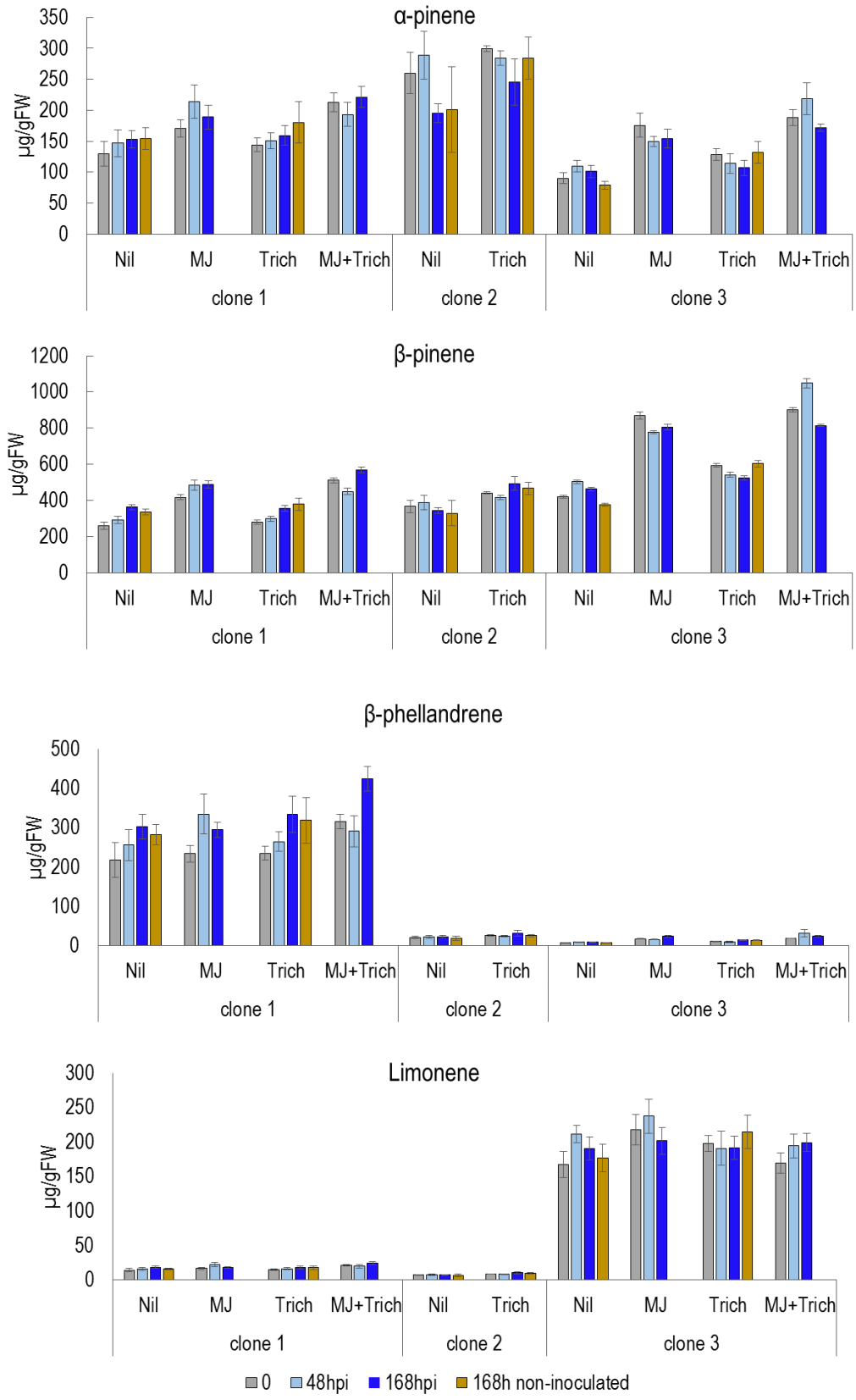
phellandrene & camphene in clone 3, compared with their respective untreated controls.

- Clone 2 was not treated with MJ at Ruakura (insufficient plants available).
- *Trichoderma* caused an increase in the concentrations of β-pinene and β-phellandrene in clone 3 compared with in the untreated control.
- *Trichoderma* did not affect terpene concentration in clones 1 & 2.
- *Trichoderma*+MJ caused in an increase in camphene and myrcene in clone 1, and increases in α-pinene, β-pinene, β-phellandrene and camphene in clone 3.

Post-inoculation effect - changes in terpenes at 48h and 168h post inoculation (hpi):

- Treatment effects cannot be differentiated from the effects of inoculation alone because of the lack of appropriate comparisons (insufficient plant material). However, the comparison of inoculated and non-inoculated plants for the control and the *Trichoderma* at 168h suggests that inoculation with *D. sapinea* did not have a strong effect on needle terpenes;
- <u>At 48hpi;</u>
    - In clone 1, concentrations of α-pinene, β-pinene, camphene, and myrcene were greater in MJ-treated plants than in the untreated control.
    - In clone 3, β-pinene, myrcene, and β-phellandrene were greater in MJ-treated plants than in the untreated control. Furthermore, α-pinene, β-pinene and β-phellandrene were greatest in cuttings treated with *Trichoderma*+MJ.
    - There was no effect of *Trichoderma* alone.
- <u>At 168hpi:</u>
    - In clone 1, α-pinene, β-pinene, camphene, myrcene, β-phellandrene and trans-caryophellene were greater in *Trichoderma*+MJ than in the untreated control.
    - In clone 2, limonene was greater in *Trichoderma*-treated cuttings than in the untreated control.
    - In clone 3, α-pinene, β-pinene, myrcene, β-phellandrene and terpinolene were greater in MJ and *Trichoderma*+MJ than in untreated controls.
    - In clone 3, β-phellandrene was greater in *Trichoderma*-treated cuttings than in the untreated controls.

Figure 3 – Needle chemistry (legend on next page)



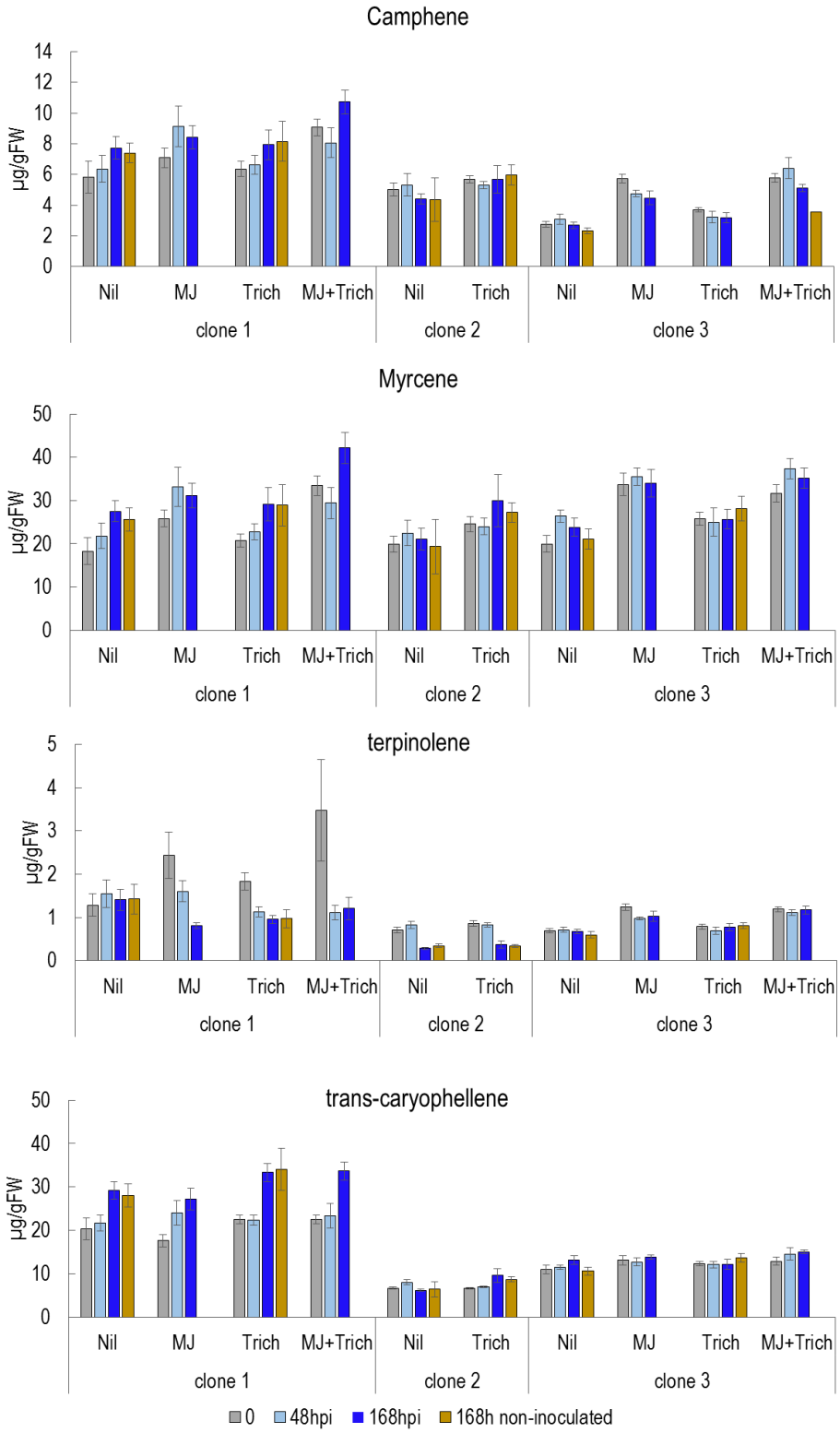


Figure 3. Selected terpenoids in needles from three *Pinus radiata* clones treated with Trichoderma or methyl jasmonate and then inoculated with *Diplodia sapinea*. Samples were taken at 0h, 48 h, or 168 h post inoculation (hpi) with *D. sapinea.* Samples were collected also from non-pathogen challenged plants at 168h for the Nil and *Trichoderma* treatments only. For clone 2 there were insufficient trees to include MJ or MJ + Trich.

Scion experiment

The effects of treatment with MJ and *Trichoderma* and the subsequent response to challenge inoculation with *P. pluvialis* on the concentrations of eight selected terpenoid compounds, the same compounds as shown for *D. sapinea* (Figure 3), are shown in Figure 4 (the details of 10 compounds are available in Appendix Table A4).

Clonal effects - comparisons between the untreated controls, were similar to those observed in the *D. sapinea* exeriment:

- β-phellandrene concentration was over 10x greater in clone 1 than in clones 2&3 for the majority of time points.
- Limonene concentration was over 10x greater in clone 3 than in clones 1&2.
- trans-caryophellene concentration was 2-4x greater in clone 1 than in clones 2&3.
- Inoculation with *P. pluvialis* did not induce a change in terpene concentration in untreated plants over the duration of the experiment (168h).

In the Scion *P. pluvialis* experiment there was very little difference in α-pinene between the clones, in contrast to the Ruakura *D. sapinea* experiment where there was a greater level of α-pinene in clone 2 than clones 1&3. This difference reflects a site variation in compound expression between the two experiments.

Treatment effects - effects of MJ and *Trichoderma* (before inoculation with *P. pluvialis*):

- MJ caused an increase in the concentrations of α -pinene, β-pinene, β-phellandrene, camphene, myrcene, & terpinolene in clone 1, and an increase in the concentrations of α –pinene, β-pinene, camphene, and myrcene in clone 3, compared with their respective untreated controls.
- MJ did not affect terpene concentration in clone 2.
- *Trichoderma* caused an increase in the concentration of β-pinene in clone 1, and an increase in β-phellandrene and trans-caryophellene in clone 2, compared with their respective untreated controls.
- *Trichoderma* caused a decrease in α –pinene, β-pinene, Limonene, myrcene and trans-caryophellene in clone 3, compared with in the untreated control.
- *Trichoderma*+MJ caused in an increase in β-pinene and myrcene in clone 1, an increase in α-pinene, β-pinene, β-phellandrene, camphene, myrcene, terpinolene and trans-caryophellene in clone 2, and an increase in α-pinene, β-pinene, camphene, and myrcene in clone 3, compared with their respective untreated controls.

There were differences in terpene concentrations between clones both within the same experiment (Scion only) and between experiments (comparison of results between Ruakura and Scion), for the same treatments.

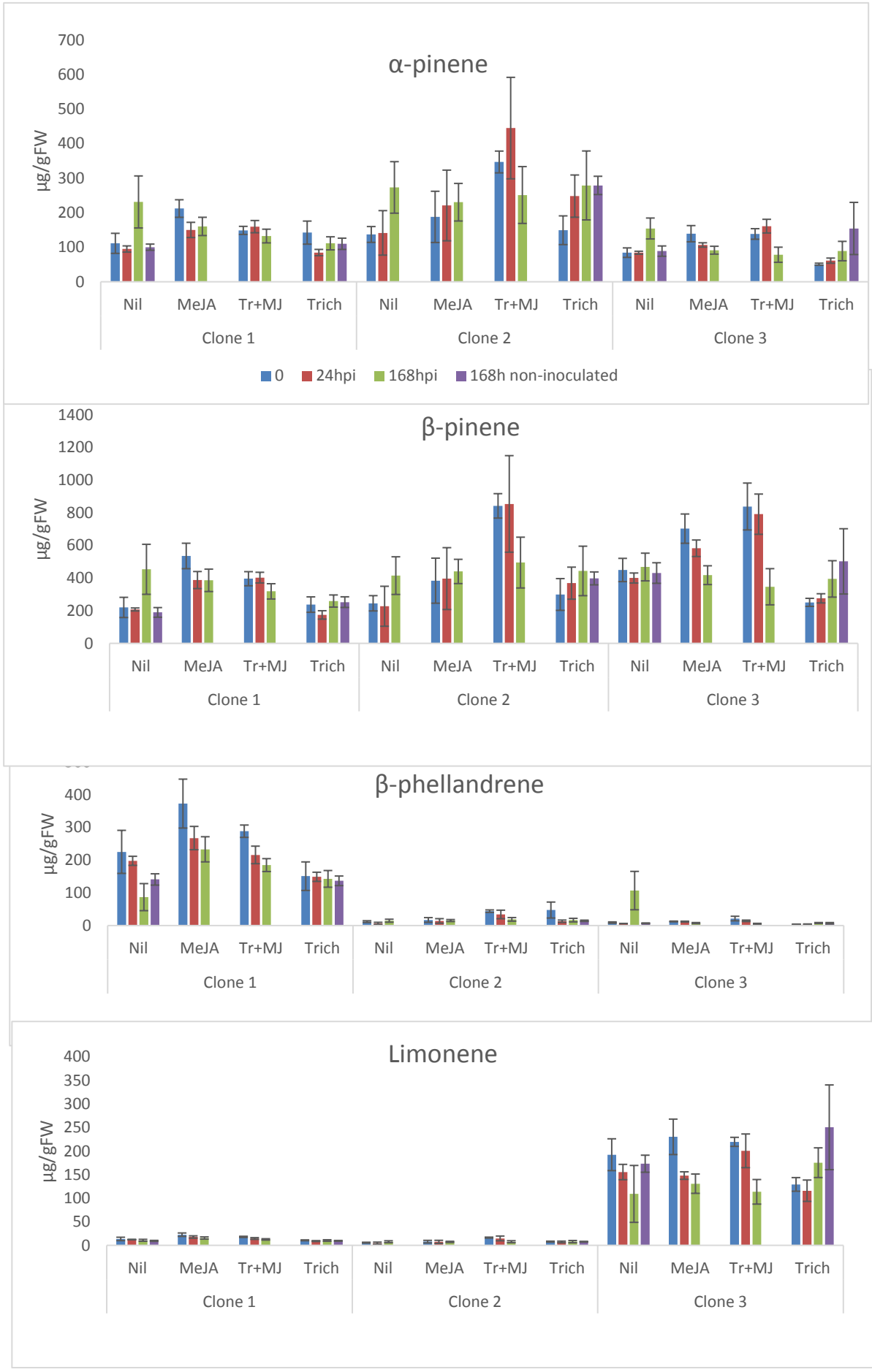
Post-inoculation effect - changes in terpenes at 24h and 168h post inoculation (hpi):

- <u>At 24hpi;</u>
    - In clone 1, concentrations of α-pinene, β-pinene, β-phellandrene, camphene, and myrcene were greater in MJ-treated plants, and there were greater concentrations of α-pinene, β-pinene, myrcene and terpinolene in the MJ-*Trichoderma* treated plants, than in their respective untreated controls.
    - There was a decrease in β-phellandrene and terpinolene for clone 1 in the*Trichoderma* alone treatment than in the untreated controls.
    - In clone 2, the concentration of α-pinene, β-pinene, camphene, myrcene and terpinolene was greater in the MJ+*Trichoderma*-treated plants than in the untreated controls.

    - There was no effect of MJ or *Trichoderma* alone in clone 2.
    - In clone 3, the concentration of α-pinene, β-pinene, camphene, myrcene, and trans-caryophyllene was greater in the MJ treated plants, and the concentration of α-pinene, β-pinene, camphene, myrcene, terpinolene and trans-caryophyllene was greater in the MJ+*Trichoderma*-treated plants than in their respective untreated controls.
    - There was a decrease in camphene and myrcene in the *Trichoderma* - treated plants for clone 3.
- <u>At 168hpi:</u>
    - In clone 1, β-phellandrene, terpinolene and trans-caryophellene were greater in the MJ and *Trichoderma*+MJ treated plants, than in the respective untreated controls.
    - There was a decrease in α-pinene in the *Trichoderma*-treated plants than in the untreated control.
    - In clone 2, terpinolene was greater in *Trichoderma*+MJ treated plants, than in the respective untreated controls.
    - There was no effect of MJ or *Trichoderma* alone in clone 2.
    - In clone 3, there was a decrease in α-pinene, β-phellandrene, camphene, terpinolene and trans-caryophellene for all three treatments (MJ, *Trichoderma*+MJ and *Trichoderma*) than in the respective untreated controls; there was also a decrease in myrcene in the *Trichoderma*+MJ treated plants.

There was little evidence to suggest that inoculation with *P. pluvialis* affects terpenoid composition in needles, the only variation was a slight increase in terpinolene in clone 2. This was similar to what was observed with *D. sapinea* where inoculation also did not affect terpenoid levels.

Figure 4. Legend on next page



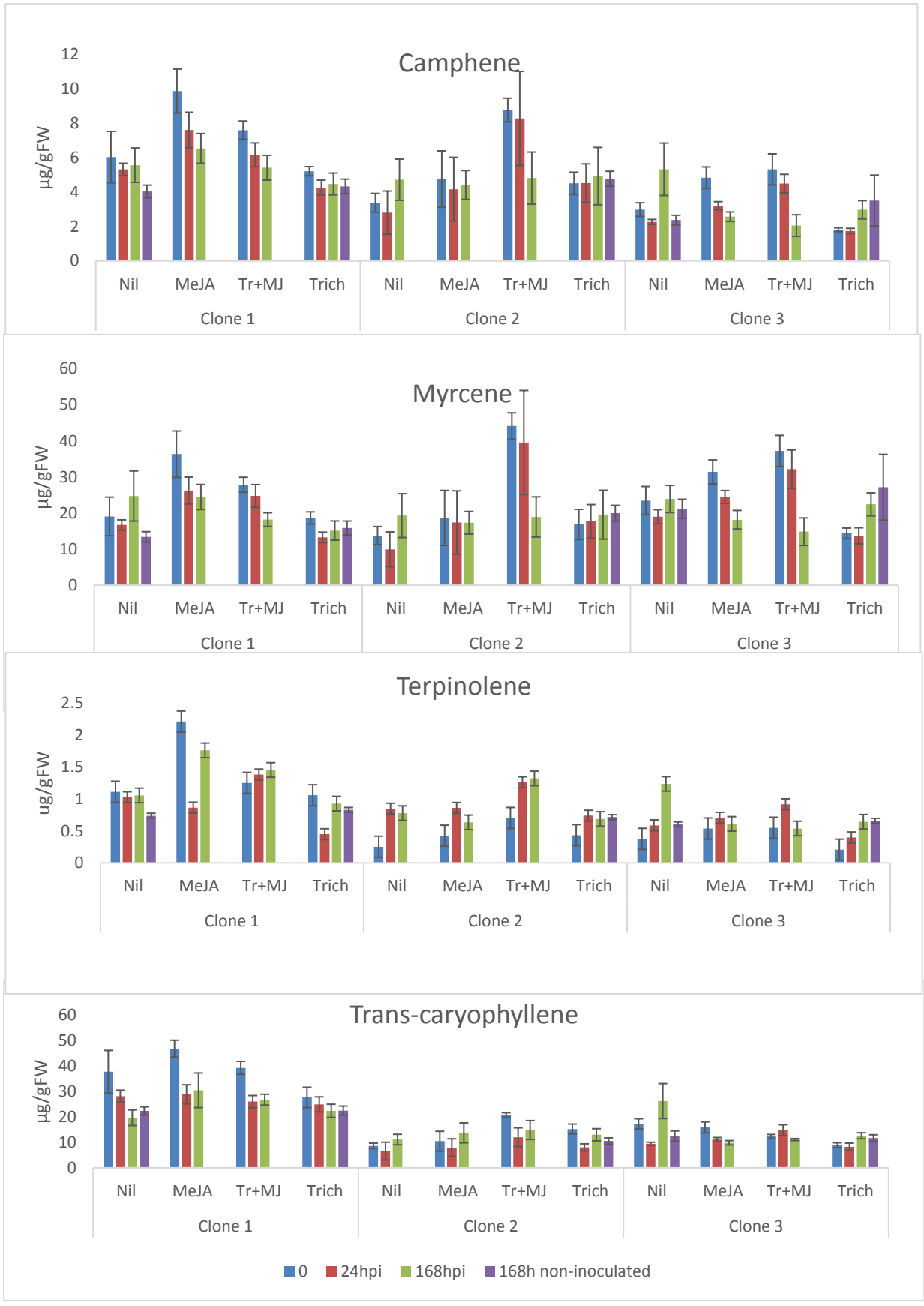


Figure 4. Selected terpenoids in needles from three *Pinus radiata* clones treated with *Trichoderma* spp. or methyl jasmonate and then inoculated with *Phytophthora pluvialis*. Samples were taken at 0h, 24 h, or 168 h post inoculation (hpi) with *P. pluvialis.* Samples were collected also from non-pathogen challenged plants at 168h for the Nil and *Trichoderma* treatments only.

Limitations of the study and future analysis

- In comparisons of the *P. pluvialis* and *D. sapinea* samples, it needs to be taken into account that the second time points were different (24 h or 48 h) between the two sets of samples, and the experiments were done at two different sites.
- The defence-related genes studied here may or may not be key indicators of defence responses for two main reasons. Firstly, some of the genes (eg. thaumatin-like protein, endochitinase) are part of gene families. Although genes showing the best expression and differential expression responses were used, there could be functional redundancy with other gene family members. Secondly, other genes could have been trialled. Our plan is to extend the analysis to include a peroxidase (the best candidate amongst >30 peroxidase genes in the genome) and one of the phenylalanine ammonium lyase (PAL) genes.
- Each of the expression analyses has only been done once so far; biological triplicates will be assessed and statistical analyses performed.
- There was insufficient trees available to include MJ and MJ+*Trichoderma* treatments on clone 2 at Ruakura.
- There were insufficient trees available to include non-challenged controls for every treatment and therefore the effects of pathogen-inoculation alone on needle chemistry must be inferred from the untreated control and the Trichoderma-treated plants only.

Conclusions and Recommendations

In conclusion:

- A set of PCR primers was developed for 33 defence-related genes of *P. radiata*. Six of these were used to analyse gene expression in pines treated with Trichoderma and/or methyl jasmonate, and challenged with *P. pluvialis* or *D. sapinea.*
- There was a high level of clone-specificity in expression of *Pinus radiata* defence-related genes. However the RNC 'resistant' clone 1 did not appear to show higher levels of induction of the six defence genes in response to *P. pluvialis,* compared to the other clones.
- Defence-related gene expression levels were generally higher in plants challenged with *P. pluvialis* than with *D. sapinea*, and induction was more pronounced. The exceptions were the limonene and alpha pinene genes, which showed the opposite pattern.
- Methyl jasmonate treatment appeared to induce higher levels of gene expression than Trichoderma treatment, but more work is required to evaluate the complex patterns seen. Further analysis of the samples will be carried out as part of an MSc project.
- There were measurable differences in terpenoid composition between the three clones. The difference in limonene and β-phellandrene concentration between the three clones is of particular note (limonene concentration was over 10x greater in clone 2 than in clones 1&3 but β-phellandrene was over 10x greater in clone 1 than in clones 2&3)
- Most of the changes in terpenoid composition appear to be driven by methyl jasmonate, however, there is evidence of a differential clonal response to *Trichoderma* and in some cases an additive effect of *Trichoderma*+MJ on terpenoid content.
- There was little evidence to suggest that inoculation with *Diplodia sapinea* or *Phytophthora pluvialis* affects any terpenoid composition in needles.

Recommendations:

- To further investigate the mechanisms that influence resistance and susceptibility in radiata pine, and how they differ between oomycete and fungal pathogens, a larger trial with methyl jasmonate treatment, and challenge with either *P. pluvialis* or *D. sapinea*, should be carried out. Ideally the trials with the two pathogens should be carried out at the same location.

- To obtain a broader perspective of plant defence, we recommend metabolite profiling and whole-genome gene expression profiling. These would build directly on the enabling technologies work of the HTHF *Phytophthora* programme.
- A more detailed investigation of the differential response of clones to *Trichoderma* and the additive effect of *Trichoderma*+MJ on terpenoid content. Other elicitors should also be considered to broaden our fundamental understanding of elicitor/endophyte interactions and their potential to affect defence biochemistry.

Acknowledgements

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Appendix Table A1 - PCR primers designed for *Pinus radiata* putative defence genes and normalisation controls

	A	B	C	D	E	F	G	H	I
1	Target Genes	ortholog		Primer	Primer	Primer	Primer	PCR product s	Designed by
2		GENE ID	FUNCTION NOTES	FWD name	FWD Seq 5' to 3'	REV name	REV Seq 5' to 3'	bp	
3	Ankyrn	PtaS25535633	Ankyrin domain	AnkyF	GCATTAGTCCGGTTGAAAATTCG	AnkyR	AAGGAATACACAATATATCTCCGT	202	Lisa Stanbra
4	**Endo Cf**	AEF59005.1	class i chitinase P contorta	EndoChF	CATCTGTGTACCCGTTTGCC	EndoChR	ACCAAATCCCCATTATTCTCAACC	113	Lisa Stanbra
5	Limonene	DR015595	cyclic terpene	Limon1F	GTGTCTAATTGAACCACTGCC	Limon1R	GATTCTAAATTCCAAGCCTCCT	207	Lisa Stanbra
6	**Limonene 2**	DR018762	cyclic terpene	Limon2F	CCTTGAACCTGTGCCTTTGT	Limon2R	CACATGGGGAAGATTGAGACAC	101	Lisa Stanbra
7	GST	PtaS15773937	detoxification	GSTF	ACTGGCATGTCATCTTTTGTTCA	GSTR	TGGGAAAATTTGTGGGCCTG	87	Lisa Stanbra
8	BglU	48934453	endoPG defence may be nonspecific?	BglucF	ACGAGAATTTGAAAGGCGGG	BglucR	AGGGGAGAAGTTAACAGGGT	104	Lisa Stanbra
9	Lipo Ox	BK24136.1	JA/ET rel LOX/unknown lipase	Lipox1F	TACCGCTCATTTCACCGTCT	Lipox1R	TCTCCGATCTGACTAGGGCT	133	Lisa Stanbra
10	Jas Met	PtaS16844814	jasmonate/SA O-methyltransferase	JasMetF	TGAAAGCTCTAAATAGTCGGTGT	JasMetR	TGAACTCTAACTACCTTGCGG	83	Lisa Stanbra
11	4CL	U12013	lignin	4clF	TGCAGAGTAAGCGCCCTATAA	4clR	GTAGGGCGTTGACAATCCAT	70	H Flint,A Wa
12	C3H	17978650	lignin	C3hF	TGGTCAACATGCAGCTTTCT	C3hR	TCAATTTGAGGAATAGGTATTTGTTC	90	Lisa Stanbra
13	C4H	AF096998	lignin	C4hF	GGGTTTCAATAACAGACACCGTCAA	C4hR	CCCAATTGGTGGAGAGTCAA	102	H Flint,A Wa
14	Cad	Z37991	lignin	CadF	GTCCGTTACAGATTTGTGGTGGATGTT	CadR	AGCTTCCCATCTCTTCAGAACCACTTC	255	H Flint,A Wa
15	CCR	AY064169	lignin	CcrF	GCGAAACAATGCCTGTATGA	CcrR	TTTTTAGTACACGATCCTCCATCA	194	H Flint,A Wa
16	**PhehyF**	HQ003814	phe to tyr phenylprop regulation?	PhehyF	CAAGATGAGGGATATTGCCACA	PhehyR	GCCAGTCCACTTGAATTTAGCA	171	Lisa Stanbra
17	**CCoAOMT**	AF036095	lignin and stilbenes first p/way step	CCoAOMTF	TTGCAGGCGTGTCTATTGAAAACAATC	CCoAOMTR	CAAATGGCTTCAACCCCATA	110	H Flint,A Wa
18	Per 1	CF666571	peroxidase	Perox1F	TTTTCTGCAATTAAGGGAGCTTT	Perox1R	TCTTTACAGTGGAGGAAAACGA	124	Lisa Stanbra
19	Per 2	DR163369	peroxidase/catalase?	Perox2F	TCATCAAGACCCTGTACATTGAC	Perox2R	TTTCGATGGAGACCTTTCAACG	150	Lisa Stanbra
20	Per 3	DR069511/DR1	peroxidase	Perox3F	ACACGCTTATATGGATTGCAGA	Perox3R	ACCACCCATCAACACAACTAT	150	Lisa Stanbra
21	Per 4	DR070948	peroxidase	Perox4F	GGAAGCCAAACTAACCCTCG	Perox4R	AGCCAGTGAAATGAAGTAGATGA	75	Lisa Stanbra
22	**α pinene**	AF543527	pinene	AlphpinF	CTTAAGCGGTCGTTGGATGT	AlphpinR	GGCCACTGGATACATATACACC	76	Lisa Stanbra
23	β pinene	AF543531	pinene may also be alpha pinene?	BetapinF	TATCCCGATGGCAGGTTCTT	BetapinR	CTCCGGCAGTGAAAGTTTCC	120	Lisa Stanbra
24	PSY 271	NZPradTrx1152	pinosylvin stilbene synthase phenolic	PSY271F	CCATGGCAATCTACCTCCCT	PSY271R	CCTGAACTGCTGTGGAGACT	121	Lisa Stanbra
25	Thaum 1	gi|284821892|(	thaumatin-like L4 P monticola	Thaum1F	AGGAAAGAGGCTATTGAAGTGA	Thaum1R	TCATCCGAGGAATGCTCTC	129	Lisa Stanbra
26	**Thaum 2**	gi|284821896|(	thaumatin-like L6 P monticola	Thaum2F	GTCGTCTTCTGCGGTTGAC	Thaum2R	TGTTGTAATTAGCACCACCAAG	124	Lisa Stanbra
27	Thaum3F	gi|116790974|(	unknown thaumatin-like Quercus	Thaum3F	AAGACCATAGTTTAAGAGCAGCC	Thaum3R	CTATCAAGTGCCCAAAGTGAA	140	Lisa Stanbra
28	SAR1F	DR050081	SA reponse/degradation?	SAR1F	GAAAAGGGTCAAAATGCGCAT	SAR1R	CTAAAACCTATATAGACGCTGCC	91	Lisa Stanbra
29	SARJAF	DR163847	SAR/aromatic aa/JA biosynthesis	SARJAF	ATTCAGGCCAGGCAGATCC	SARJAF	AGCCTTTACTAAGCTAAAACCAG	83	Lisa Stanbra
30	MAPK 26	At3G45640	signaling	MPK26F	GTGGGCGATCGGAATCCTC	MPK26R	AGAACCCCAAACCTGCAGAG	80	Rosie Bradsh
31	StilB	BAA94593.1	stilbene	StilbF	TCTTATAGCTATTCGACCACACCA	StilbR	CCCCATGTCTAGTACCAAACC	85	Lisa Stanbra
32	WRKY	ABS18435.1	WRKY6 TF defence/senescence	WRKY6F	TTTGCAGAGGGATTCAAATTCTTT	WRKY6R	ACTCCTGCACTTGTAATGACC	111	Lisa Stanbra
33	Chi4F	AAS83984.1	class IV chitinase? Chi4F	Chi4F	GGGAGGTGAATAGCAGAGTGA	Chi4R	AAAGACAGATTTAACACGAAACA	93	Lisa Stanbra
34	MPK168	NZPr096168 CO1	MPK3 Mitogen activated protein kinase	MPK168F	ACTGTCGACAAGCACACACA	MPK168R	AGCCGAGGGTCTGGTTACTC	99	Rosie Bradsh
35	PinoSS	NZPradTrx1110	pinosylvin synthase	PSY72F	AGAGATTCTTATAGCTATTCGACCA	PSY72R	AAACCCTGGACAACAAATCTCA	75	Lisa Stanbra
36									
37	Normalisation control gene candidates								
38	**Actin**		Actin	ActinF	TGTAGCCCTTGACTATGAGC	ActinR	AGGGACCTGACTCCTCATAC	449	Lisa Stanbra
39	GPDH		glyceraldehyde-3-phosphate dehydrogen	GPDF	ATTTGATGGGTCTTTTGTGGAC	GPDR	CCTAAACACACTGGGTTGCC	149	Lisa Stanbra
40	Rubisco		ribulose-1,5-bisphosphate carboxylase/o	RBCF	CCAGTGACTTTCGTTCTACCA	RBCR	TGAAATCCCATATCTCCGCTCA	127	Lisa Stanbra
41	**Tef1**		translation elongation factor 1-alpha	TEF1F	GCATTTTAGTAATTTGGGCGGG	TEF1R	AGACGTTGCAATGGTCTTTGA	104	Lisa Stanbra

Appendix Table A2 - Defence gene expression pre-screen rounds 1 (with 33 primer sets) & 2 (with 14 primer sets)

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
1						Round 1 expression ratios Scion rep 1 clone 21-24							Round 2 expression ratios Scion rep 1						
2	Target Genes	Gene model not good	Non-specific amplifc ation	no amplific ation	expression level mean of 10 samples	Tricho/nil pathogen	Tricho/nil NO pathogen	pathogen/ nil Tricho	pathogen/ nil NO Tricho	MeJa/nil mean of 3 times	Use for round 2 screen (14)	**Target Genes**	clone 1 Tri&MeJa 24h/0h	clone 2 Tri&MeJa 24h/0h	clone 3 Tri&MeJa 24h/0h	clone 2 Tri 168h/24 h	clone 3 Tri 168h/24 h	large diffs b/t tree clones	use for final (6 genes)
3	Ankyrn				3.107	1.471	0.199	1.078	0.146	0.62		**Ankyrn**							
4	**Endo Cf**				1.352	1.125	3.757	3.744	12.501	44.98	Y	**Endo Cf**	12.443	9.564	39.854	2.935	0.090	Y	Y
5	Limonene				0.150	0.118	n/a	68.006	n/a	0.39	Y	**Limonene**	xx	2.194	2.796	129.218	8.903	Y	
6	**Limonene 2**			low	0.025	1.509	1.375	0.659	0.601	0.84	Y	**Limonene 2**	5.017	0.733	0.241	1.465	2.958	Y	Y
7	GST	X			0.078	0.915	0.472	0.996	0.514	0.96		**GST**							
8	BglU				6.757	0.614	0.211	21.472	7.373	9.91	Y	**BglU**	28.298	12.608	45.443	5.627	0.110	Y	
9	Lipo Ox				0.486	0.671	2.392	0.842	2.999	0.88		**Lipo Ox**							
10	Jas Met				0.806	0.510	0.497	6.201	6.044	2.43	Y	**Jas Met**	9.220	0.187	11.566	8.067	0.241	Y	
11	4CL		X		1.665	0.983	2.084	0.830	1.759	1.47		**4CL**							
12	C3H				1.572	1.497	0.582	1.519	0.591	1.12		**C3H**							
13	C4H				0.262	0.620	4.162	2.269	15.217	0.86		**C4H**							
14	Cad				0.137	0.714	1.449	1.427	2.898	2.33	Y	**Cad**	0.985	1.079	0.864	1.214	0.255		
15	CCR		X		1.496	0.534	0.534	2.798	2.797	0.72		**CCR**							
16	**PhehyF**				0.936	1.130	0.142	0.094	0.083	6.79	Y	**PhehyF**	37.726	12.634	24.054	0.355	0.088	Y	Y
17	**CCoAOMT**				7.006	0.867	1.726	1.163	2.316	1.61	Y	**CCoAOMT**	1.094	3.334	1.758	1.506	0.361		Y
18	Per 1				0.208	1.006	1.612	0.566	0.907	1.28	Y	**Per 1**	3.931	3.608	8.523	3.126	0.101	Y	
19	Per 2	X	X		79.322	1.005	1.612	0.566	0.907	1.12		**Per 2**							
20	Per 3				0.286	0.495	0.303	2.513	1.540	1.60	Y	**Per 3**	1.060	0.864	2.066	1.935	0.239		
21	Per 4	X		low	0.005	n/a	1.694	n/a	0.116	15.88		**Per 4**							
22	**α pinene**			low	0.006	1.466	13.234	5.198	46.930	0.94	Y	**α pinene**	0.655	0.374	0.708	1.098	0.313		Y
23	β pinene	(x)		low	0.004	0.254	0.386	3.829	5.822	1.31	Y	**β pinene**	0.951	0.733	0.326	1.465	0.313		
24	PSY 271			low	0.006	0.443	0.251	0.504	0.286	1.84		**PSY 271**							
25	Thaum 1				0.717	0.545	2.595	2.980	14.203	5.15	Y	**Thaum 1**	1.761	7.839	3.607	6.471	1.551	Y	
26	**Thaum 2**				0.486	1.021	0.401	2.630	1.033	4.17	Y	**Thaum 2**	6.150	6.080	19.120	3.205	0.023	Y	Y
27	Thaum3F				0.215	0.237	3.165	0.398	5.311	0.44		**Thaum3F**							
28	SAR1F	X			0.305	0.931	0.600	0.691	0.445	0.61		**SAR1F**							
29	SARJAF				4.589	2.009	6.196	0.852	2.626	0.46		**SARJAF**							
30	MAPK 26				0.416	0.784	0.619	1.071	0.846	1.46		**MAPK 26**							
31	StilB			low	0.012	0.220	0.000	202.161	0.000	3.21		**StilB**							
32	WRKY				0.809	0.230	0.065	3.116	0.877	2.04		**WRKY**							
33	Chi4F			X								**Chi4F**							
34	MPK168		X	X								**MPK168**							
35	PinoSS			X								**PinoSS**							

Appendix Table A3 - Concentrations of terpene compounds (µg/gFwt) in needle tissues (Ruakura).

Clone	Time	Treatment	α-Pinene	β-Pinene	Camphene	Limonene *	Myrcene	α-Phellandrene *	β-Phellandrene *	α-Terpinene	Terpinolene *	trans-Caryophyllene
1	0h	*Control*	129.5 ± 20.24 ef	259.1 ± 42.09 e	5.8 ± 1.06 bc	13.9 ± 2.46 c	18.2 ± 3.12 d	5.2 ± 1.1 b	217.2 ± 43.93 b	0.4 ± 0.07 b	1.2 ± 0.26 cd	20.3 ± 2.5 ab
		MeJA	170.6 ± 13.55 cde	416.3 ± 22.75 cd	7 ± 0.65 b	16.6 ± 1.43 bc	25.8 ± 1.91 bc	5.4 ± 0.61 ab	233.8 ± 21.47 ab	0.4 ± 0.02 b	2.4 ± 0.53 ab	17.6 ± 1.41 b
		Trich	144 ± 11.05 de	278.9 ± 28.88 e	6.3 ± 0.49 bc	14.8 ± 1.06 c	20.7 ± 1.51 cd	5.7 ± 0.42 ab	234.8 ± 16.95 ab	0.4 ± 0.03 b	1.8 ± 0.2 bc	22.5 ± 1.06 a
		Trich+MeJA	213.1 ± 15.17 bc	510.8 ± 40.93 bc	9 ± 0.55 a	21.1 ± 1.25 b	33.4 ± 2.27 a	7.5 ± 0.41 a	315.6 ± 18.53 a	0.5 ± 0.07 a	3.4 ± 1.16 a	22.5 ± 1.07 a
2		*Control*	260.1 ± 33.78 ab	365.6 ± 46.29 de	5 ± 0.43 cd	7.1 ± 0.48 d	19.9 ± 1.83 cd	0.2 ± 0.01 cd	20.5 ± 2.03 cd	0.1 ± 0 c	0.7 ± 0.06 e	6.7 ± 0.28 d
		Trich	299.6 ± 4.85 a	441.7 ± 38.88 cd	5.6 ± 0.23 bc	8.4 ± 0.49 d	24.5 ± 1.73 cd	0.3 ± 0.01 cd	25 ± 1.89 c	0.1 ± 0.01 c	0.8 ± 0.06 de	6.7 ± 0.17 d
3		*Control*	90.4 ± 8.86 f	419.6 ± 30.88 cd	2.7 ± 0.19 e	166.9 ± 18.82 a	19.9 ± 1.95 cd	nd	6.3 ± 0.59 f	nd	0.6 ± 0.05 e	10.9 ± 1.03 c
		MeJA	175.7 ± 19.68 cd	868.9 ± 70.17 a	5.7 ± 0.28 bc	217.8 ± 22.28 a	33.7 ± 2.62 a	0.3 ± 0.03 c	16.8 ± 1.03 d	0.2 ± 0 c	1.2 ± 0.08 cd	13 ± 1.01 c
		Trich	128.3 ± 9.2 ef	593.4 ± 46.71 b	3.6 ± 0.14 de	197.7 ± 11.76 a	25.8 ± 1.51 bc	0.2 ± 0.01 d	10.2 ± 0.88 e	0.2 ± 0.03 c	0.7 ± 0.06 de	12.3 ± 0.56 c
		Trich+MeJA	187.8 ± 13.12 cd	901.1 ± 23.71 a	5.7 ± 0.29 bc	169.4 ± 14.59 a	31.6 ± 2.02 ab	0.3 ± 0.01 c	18.1 ± 0.59 cd	0.2 ± 0 c	1.1 ± 0.05 cd	12.8 ± 0.92 c
1	48hpi	*Control*	146.9 ± 21.65 cd	290.8 ± 40.98 d	6.3 ± 0.87 bc	16 ± 1.91 b	21.8 ± 2.88 c	5.2 ± 0.71 a	256.2 ± 39.77 a	0.1 ± 0.02 bc	1.5 ± 0.31 a	21.6 ± 1.88 a
		MeJA	214.1 ± 27.25 b	484.7 ± 64.89 c	9.1 ± 1.32 a	21.7 ± 3.23 b	33.1 ± 4.5 ab	6.1 ± 1.03 a	334.5 ± 50.37 a	0.3 ± 0.09 a	1.6 ± 0.24 a	24 ± 2.81 a
		Trich	150.7 ± 12.46 cd	297.4 ± 28.17 d	6.6 ± 0.6 bc	16.2 ± 1.5 b	22.7 ± 1.9 c	4.8 ± 0.45 a	264.5 ± 25.67 a	0.3 ± 0.03 ab	1.1 ± 0.11 ab	22.3 ± 1.18 a
		Trich+MeJA	193.1 ± 19.26 bc	449.3 ± 55.62 cd	8 ± 0.97 ab	19.5 ± 2.51 b	29.4 ± 3.57 abc	5.3 ± 0.66 a	290.6 ± 40.36 a	0.3 ± 0.04 a	1.1 ± 0.16 ab	23.3 ± 2.79 a
2		*Control*	288.9 ± 38.97 a	386.8 ± 49.66 cd	5.3 ± 0.71 cd	7.7 ± 0.96 c	22.4 ± 2.91 c	0.3 ± 0.06 c	21.8 ± 3.26 bc	0.1 ± 0.01 c	0.8 ± 0.08 bcd	8 ± 0.62 cd
		Trich	284.2 ± 11.92 a	416.3 ± 36.58 cd	5.3 ± 0.22 cd	8.1 ± 0.48 c	24 ± 1.97 c	0.3 ± 0.01 bc	23.2 ± 2.42 bc	0.1 ± 0.01 c	0.8 ± 0.04 bcd	6.9 ± 0.13 d
3		*Control*	109.9 ± 9.71 d	502.4 ± 41.69 c	3 ± 0.31 d	211.2 ± 12.66 a	26.4 ± 1.4 bc	0.2 ± 0.01 c	8.4 ± 1.09 d	0.1 ± 0 bc	0.7 ± 0.06 cd	11.4 ± 0.49 bcd
		MeJA	149.2 ± 8.29 cd	777.5 ± 25.48 b	4.7 ± 0.22 cd	237.1 ± 24.48 a	35.5 ± 2.07 a	0.2 ± 0.01 c	15.1 ± 0.41 c	0.1 ± 0 bc	0.9 ± 0.03 bc	12.7 ± 0.92 bc
		Trich	113.8 ± 15.98 d	542 ± 85.67 c	3.2 ± 0.37 d	190.6 ± 24.62 a	25 ± 3.28 bc	0.2 ± 0.02 c	8.8 ± 1.1 d	0.1 ± 0 abc	0.6 ± 0.08 d	12 ± 0.81 bc
		Trich+MeJA	218.6 ± 25.62 b	1048 ± 90.43 a	6.4 ± 0.67 bc	194.1 ± 17.58 a	37.2 ± 2.35 a	0.5 ± 0.18 b	31.6 ± 9.11 b	0.1 ± 0.03 bc	1.1 ± 0.06 ab	14.5 ± 1.44 b
1	168hpi	*Control*	152.9 ± 14.07 cde	361.9 ± 28.72 cd	7.7 ± 0.73 b	18.2 ± 1.39 bc	27.5 ± 2.46 bcd	6.9 ± 0.73 ab	301.9 ± 31.58 b	0.4 ± 0.05 a	1.4 ± 0.24 a	29.1 ± 1.96 bc
		MeJA	188.9 ± 19.74 bc	489.2 ± 42.04 bcd	8.4 ± 0.76 b	17.7 ± 1.35 c	31.1 ± 2.8 bc	6.6 ± 0.49 b	294.8 ± 19.57 b	0.5 ± 0.04 a	0.8 ± 0.06 bcd	27.1 ± 2.53 c
		Trich	159.5 ± 15.94 c	354.6 ± 38.77 cd	7.9 ± 0.95 b	17.8 ± 2.17 c	29 ± 3.85 bcd	7.4 ± 0.99 ab	333.9 ± 45.82 ab	0.4 ± 0.04 a	0.9 ± 0.09 abcd	33.3 ± 2.1 ab
		Trich+MeJA	221.5 ± 16.68 ab	568.3 ± 63.74 b	10.7 ± 0.78 a	24.3 ± 2.28 b	42.1 ± 3.59 a	9.2 ± 0.66 a	424.7 ± 31.7 a	0.5 ± 0.05 a	1.2 ± 0.26 ab	33.7 ± 2.08 a
2		*Control*	195.1 ± 15.7 abc	342.8 ± 39.98 d	4.3 ± 0.33 cd	7.2 ± 0.66 e	21 ± 2.45 d	0.2 ± 0.01 cd	22.1 ± 2.38 c	0.1 ± 0 b	0.2 ± 0.02 e	6.1 ± 0.22 f
		Trich	245.7 ± 37.32 a	492.7 ± 92.3 bcd	5.6 ± 0.9 c	10.2 ± 1.87 d	30 ± 6.02 bcd	0.3 ± 0.06 c	30.8 ± 7.09 c	0.8 ± 0.82 b	0.3 ± 0.07 e	9.5 ± 1.52 ef
3		*Control*	101 ± 10.17 e	462.9 ± 45.03 bcd	2.6 ± 0.19 d	190.4 ± 16.89 a	23.8 ± 2.09 cd	0.1 ± 0.01 e	8.3 ± 0.97 e	0.1 ± 0.01 b	0.6 ± 0.04 d	13.1 ± 1.04 de
		MeJA	154 ± 15.42 cd	805.4 ± 179.65 a	4.4 ± 0.44 cd	201.4 ± 19.21 a	34 ± 3.22 ab	0.2 ± 0.02 c	22.7 ± 2.26 c	0.1 ± 0.01 b	1 ± 0.11 abc	13.8 ± 0.55 de
		Trich	107 ± 11.93 de	522.6 ± 47.95 bc	3.1 ± 0.31 d	191.8 ± 16.74 a	25.7 ± 2.23 bcd	0.2 ± 0.02 de	13.4 ± 1.05 d	0.1 ± 0 b	0.7 ± 0.07 cd	12.1 ± 1.17 de
		Trich+MeJA	171.8 ± 5.72 bc	813.7 ± 62.46 a	5 ± 0.23 c	199.1 ± 13.22 a	35.1 ± 2.38 ab	0.3 ± 0.02 c	23.6 ± 2.28 c	0.1 ± 0.01 b	1.1 ± 0.09 a	15 ± 0.41 d
1	168 non-inoculated	*Control*	154.5 ± 17.44	335.1 ± 22.32	7.3 ± 0.63	16 ± 1	25.5 ± 2.71	6.4 ± 0.62	282.1 ± 25.94	0.5±0.06	1.4±0.34	28 ± 2.64
		Trich	180.5 ± 33.82	378.6 ± 67.77	8.1 ± 1.3	17.5 ± 2.51	28.8 ± 4.82	6.9 ± 1.16	318.3 ± 57.49	0.4±0.06	0.9±0.21	34 ± 4.79
2		*Control*	200.9 ± 69.03	329.1 ± 111.51	4.3 ± 1.42	6.6 ± 2.08	19.3 ± 6.32	0.2 ± 0.03	17.5 ± 5.7	0.1±0.01	0.3±0.04	6.3 ± 1.76
		Trich	283.9 ± 33.98	466.1 ± 49.93	5.9 ± 0.65	9.6 ± 0.98	27.2 ± 2.32	0.3 ± 0.03	24.9 ± 2.68	0.1±0	0.3±0.03	8.6 ± 0.59
3		*Control*	79 ± 6.59	375.6 ± 41.12	2.3 ± 0.18	176.5 ± 19.72	21.1 ± 2.4	0.1 ± 0.01	6.5 ± 0.94	0±0	0.5±0.07	10.6 ± 0.92
		Trich	132.4 ± 17.58	603.5 ± 61.26	3.5 ± 0.34	214.5 ± 24.24	28.1 ± 2.9	0.2 ± 0.02	12.8 ± 1.17	0.1±0.02	0.8±0.06	13.7 ± 1.02

Values for each compound within each time point that are followed by the same letter are not significantly different (P<0.05) *Data is log transformed.

Appendix Table A4 - Concentrations of terpene compounds (µg/gFwt) in needle tissues (Scion).

Clone	Time	Treatment	α-Pinene	β-Pinene	Camphene	Myrcene	α-Phellandrene	α-Terpinene	Limonene*	β-phellandrene*	Terpinolene*	trans-Caryophyllene
1	0h	*MeJA*	212.1 ± 25.41 B	535.2 ± 77.95 AB	9.9 ± 1.28 A	36.3 ± 6.43 AB	8.2 ± 1.33A	0.4 ± 0.03ABC	22.5 ± 3.65C	372.8 ± 74.77A	2.2 ± 0.64A	46.8 ± 3.34A
		Trich+MeJA	148.8 ± 11.7 BCD	395.8 ± 43.19 A	7.6 ± 0.53 AB	27.8 ± 2.08 BCD	6.8 ± 0.46A	0.5 ± 0.03AB	17.9 ± 1.23CD	288.4 ± 18.83A	1.2 ± 0.11AB	39.3 ± 2.54A
		Control	111.3 ± 29.02 CDE	220 ± 61.85 CD	6 ± 1.49 BC	19.1 ± 5.34 DE	5.8 ± 1.52AB	0.6 ± 0.14A	13.6 ± 3.59DEF	225.3 ± 65.79AB	1.1 ± 0.38BC	37.7 ± 8.41A
		Trich	142.4 ± 33.44 BCDE	237.4 ± 47.2 D	5.2 ± 0.27 BCD	18.6 ± 1.68 DE	3.6 ± 1.1B	0.3 ± 0.02BCD	10.6 ± 0.91EF	151 ± 43.61B	1.1 ± 0.22BC	27.7 ± 4B
2		*MeJA*	187.9 ± 74.19 BC	383.8 ± 137.92 BC	4.7 ± 1.64 CD	18.7 ± 7.62 DE	0.4 ± 0.1CDE	0 ± 0	7.8 ± 2.75G	17.2 ± 7.12D	0.4 ± 0.16EFG	10.4 ± 3.91D
		Trich+MeJA	347 ± 31.53 A	841.9 ± 74.87 CD	8.8 ± 0.69 A	44.1 ± 3.68 A	0.6 ± 0.04CD	0.3 ± 0.03CD	16.4 ± 0.96CDE	44 ± 3.79C	0.7 ± 0.04BCD	20.7 ± 0.93BC
		Control	137.1 ± 22.9 BCDE	245.3 ± 46.81 D	3.4 ± 0.54 DE	13.8 ± 2.51 E	0.2 ± 0.02E	0.1 ± 0.02D	5.7 ± 0.85G	12.1 ± 2.67D	0.3 ± 0.04FG	8.6 ± 1.05D
		Trich	149.2 ± 41.61 BCDE	299.2 ± 97.23 D	4.5 ± 0.65 CDE	16.9 ± 4.16 DE	1.2 ± 0.86C	0.3 ± 0.02CD	7.9 ± 0.94FG	47.5 ± 24.4C	0.4 ± 0.04DEF	15.2 ± 1.95CD
3		*MeJA*	139.3 ± 23.47 BCDE	702.5 ± 89.61 CD	4.8 ± 0.62 CD	31.4 ± 3.3 BC	0.3 ± 0.02DE	0 ± 0	230.1 ± 37.59A	12.6 ± 1.12D	0.5 ± 0.08CDE	15.9 ± 2.16CD
		Trich+MeJA	138.5 ± 15.29 BCDE	837.9 ± 143.59 A	5.3 ± 0.9 BCD	37.2 ± 4.33 AB	0.3 ± 0.06CDE	0.2 ± 0.04D	219.5 ± 9.57A	21.4 ± 6.98CD	0.5 ± 0.09CDE	12.3 ± 0.78CD
		Control	84.3 ± 13.74 DE	449.2 ± 71.21 D	3 ± 0.4 DE	23.5 ± 3.88 CDE	0.2 ± 0.02E	0 ± 0	192.2 ± 33.73AB	9.2 ± 2.02DE	0.4 ± 0.06DEFG	17.2 ± 2.02CD
		Trich	50.8 ± 3.06 E	251 ± 24.68 CD	1.8 ± 0.11 E	14.4 ± 1.47 E	0 ± 0	0 ± 0	129.2 ± 14.45B	4 ± 0.37E	0.2 ± 0.01G	8.9 ± 0.98D
1	24h	*MeJA*	150.1 ± 22.06 BC	387.6 ± 52.23 AB	7.6 ± 1.03 AB	26.2 ± 3.72 ABC	6.1 ± 0.78A	0.5 ± 0.07A	17.7 ± 2.5B	267.4 ± 35.62A	0.9 ± 0.11AB	28.9 ± 3.75A
		Trich+MeJA	159.7 ± 17.73 BC	402.5 ± 32.66 A	6.2 ± 0.69 ABC	24.7 ± 3.15 ABC	5.1 ± 0.62A	0.4 ± 0.04ABC	14.5 ± 1.8B	215.9 ± 26.82A	1.4 ± 0.16A	26 ± 2.39A
		Control	94.9 ± 9.03 BC	207.5 ± 9.24 BC	5.3 ± 0.35 ABCD	16.7 ± 1.42 BC	4.6 ± 0.31A	0.5 ± 0.06AB	12.2 ± 0.59BC	197.8 ± 13.85A	1 ± 0.51ABC	28.1 ± 2.38A
		Trich	84.7 ± 9.04 BC	174 ± 25.74 BC	4.3 ± 0.43 CDE	13.3 ± 1.46 C	3.9 ± 0.39BC	0.3 ± 0.04ABC	9.1 ± 0.94BCD	149 ± 14.02A	0.4 ± 0.06CD	24.9 ± 2.95A
2		*MeJA*	221 ± 102.35 BC	396.6 ± 189.51 BC	4.2 ± 1.85 CDE	17.4 ± 8.77 BC	0.3 ± 0.12B	0 ± 0	7.4 ± 3.26DE	14.1 ± 6.97CD	0.9 ± 0.39ABCD	7.9 ± 3.46BC
		Trich+MeJA	445.1 ± 146.96 A	853.9 ± 295.68 BC	8.3 ± 2.73 A	39.5 ± 14.43 A	0.5 ± 0.16BC	0.3 ± 0.08BC	14.7 ± 5.02BC	34 ± 13.02B	1.3 ± 0.42AB	12 ± 3.69BC
		Control	141.6 ± 64.45 BC	227.1 ± 122.44 C	2.8 ± 1.25 DE	10 ± 4.83 C	0 ± 0	0 ± 0	4.7 ± 2.1E	6.7 ± 3.26E	0.8 ± 0.23ABCD	6.6 ± 3.48C
		Trich	248.1 ± 61.18 B	368.6 ± 98.09 C	4.5 ± 1.12 BCDE	17.7 ± 4.65 BC	0.2 ± 0.07	0.2 ± 0.02C	7.1 ± 1.77CDE	13.2 ± 3.62BC	0.7 ± 0.16BCD	8 ± 1.42BC
3		*MeJA*	106.6 ± 6.29 BC	582.3 ± 50.52 BC	3.2 ± 0.24 CDE	24.5 ± 1.78 ABC	0.2 ± 0.01BC	0 ± 0	147.9 ± 7.99A	12.1 ± 1.26BC	0.7 ± 0.05BCD	11.1 ± 0.8BC
		Trich+MeJA	161.1 ± 19.79 BC	790.9 ± 123.24 A	4.5 ± 0.54 BCDE	32.1 ± 5.36 AB	0.3 ± 0.03BC	0 ± 0	200.4 ± 35.71A	14.4 ± 2.17BC	0.9 ± 0.15AB	14.8 ± 2.13B
		Control	84.1 ± 4.29 BC	400 ± 30.74 C	2.3 ± 0.14 DE	19 ± 1.93 BC	0 ± 0	0 ± 0	155.5 ± 16.29A	6 ± 0.5CDE	0.6 ± 0.04BCD	9.4 ± 0.65BC
		Trich	61.2 ± 7.47 C	275.5 ± 28.31 BC	1.7 ± 0.15 E	13.7 ± 2.21 C	0 ± 0	0 ± 0	115.8 ± 22.63A	4.3 ± 0.59DE	0.4 ± 0.06D	8.3 ± 1.43BC
1	168h	*MeJA*	138.7 ± 54.58 BC	369.9 ± 115.98 A	5.5 ± 1.08 AB	20.5 ± 5.18 A	5.8 ± 0.94A	0.5 ± 0.13A	15.6 ± 2.3BC	233.1 ± 38.43A	1.8 ± 0.5AB	22.9 ± 3.11BC
		Trich	176.4 ± 54.58 ABC	448.2 ± 115.98 A	7 ± 1.08 A	21.9 ± 5.18 A	4.5 ± 0.46A	0.3 ± 0.04B	12.6 ± 1.4B	184.8 ± 19.72A	1.5 ± 0.28A	32.3 ± 3.11A
		MeJA	99.9 ± 54.58 C	175.7 ± 115.98 A	4.1 ± 1.08 AB	12.9 ± 5.18 A	2 ± 1.14A	0.3 ± 0.07AB	10.7 ± 2.02BC	86.9 ± 41.39A	1.1 ± 0.17AB	23.1 ± 3.11BC
		Trich	125.8 ± 54.58 BC	277.6 ± 115.98 A	4.8 ± 1.08 AB	17.4 ± 5.18 A	3.6 ± 0.65A	0 ± 0	10.2 ± 1.61BC	142.7 ± 25.54A	0.9 ± 0.19AB	24.5 ± 3.11AB
2		*MeJA*	300.2 ± 77.19 AB	503.4 ± 164.01 A	5.2 ± 1.52 AB	18.6 ± 7.32 A	0 ± 0	0 ± 0	7.3 ± 1.18BC	15.4 ± 3.05BC	0.6 ± 0.15B	19 ± 4.4BCD
		Trich+MeJA	251.3 ± 54.58 ABC	494.6 ± 115.98 A	4.8 ± 1.08 AB	18.9 ± 5.18 A	0.4 ± 0.03B	0 ± 0	7.9 ± 2.38C	16.7 ± 5.44B	0.7 ± 0.25AB	14.8 ± 3.11CD
		Control	273.2 ± 54.58 AB	414.7 ± 115.98 A	4.7 ± 1.08 AB	19.3 ± 5.18 A	0.3 ± 0.07BC	0 ± 0	7.7 ± 2.08C	18.9 ± 5.47BCD	1.3 ± 0.58B	11.1 ± 3.11D
		Trich	309.6 ± 54.58 A	432.6 ± 115.98 A	5.2 ± 1.08 AB	20 ± 5.18 A	0 ± 0	0 ± 0	7.7 ± 1.98C	14.8 ± 4.49BC	0.8 ± 0.22B	10.6 ± 3.11D
3		*MeJA*	106.1 ± 54.58 C	503.8 ± 115.96 A	2.9 ± 1.08 B	20.9 ± 5.18 A	0.2 ± 0.02BC	0 ± 0	130.8 ± 20.47A	7.5 ± 1.72BCD	0.6 ± 0.06B	10.5 ± 3.11D
		Trich+MeJA	98.8 ± 77.19 BC	408 ± 164.01 A	2.5 ± 1.52 B	16.1 ± 7.32 A	0 ± 0	0 ± 0	113.5 ± 26.07A	5.1 ± 1.66D	0.5 ± 0.08AB	11.3 ± 4.4D
		Control	94.5 ± 54.58 C	399 ± 115.98 A	2.5 ± 1.08 B	21.4 ± 5.18 A	4.9 ± 0.31C	0.4 ± 0.05	109.1 ± 60.33A	106.9 ± 58.69CD	1.2 ± 0.2B	12.6 ± 3.11D
		Trich	139.2 ± 54.58 BC	418.7 ± 115.98 A	3.8 ± 1.25 AB	27.6 ± 5.98 A	0.2 ± 0.03BC	0 ± 0	175.3 ± 31.5A	7.8 ± 1.4CD	0.6 ± 0.11B	14 ± 3.59CD
1	168 - non inoculated	*Trich*	97.1 ± 5.9	228.8 ± 14.23	4.2 ± 0.25	14.8 ± 1.08	3.7 ± 0.37	0.3 ± 0.02	9.9 ± 0.89	148.7 ± 15.91	1 ± 0.23	23.5 ± 1.61
		Control	126.4 ± 17.3	256 ± 36.47	5.2 ± 0.78	18.1 ± 3.02	4.7 ± 0.86	0.3 ± 0.05	12 ± 2.09	189 ± 34.7	1.4 ± 0.54	28.6 ± 4.78
2		*MeJA*	192.5 ± 27.9	365.5 ± 52.48	3.7 ± 0.635	15.8 ± 2.87	0.4 ± 0	0 ± 0	6.8 ± 1.09	15.9 ± 2.79	0.5 ± 0.16	14.3 ± 2.05
		Trich+MeJA	354.1 ± 47.4	707.8 ± 85.041	6.8 ± 0.84	35.7 ± 4.82	0.4 ± 0.03	0.2 ± 0.03	12.6 ± 1.52	35.3 ± 5.02	1.1 ± 0.16	15.1 ± 1.6
		Control	160.6 ± 1.7	377.2 ± 13.21	3.6 ± 0.09	16 ± 0.12	0 ± 0	0 ± 0	6.9 ± 0.21	13.8 ± 0.35	0.7 ± 0.19	8.5 ± 1.59
		Trich	304.4 ± 39.5	451.9 ± 45.81	5.3 ± 0.65	23.2 ± 2.93	0.3 ± 0.01	0 ± 0	8.8 ± 0.98	17.4 ± 2.37	0.8 ± 0.13	11.5 ± 1.6
3		*Trich*	74.6 ± 13.8	339.2 ± 59.15	2 ± 0.39	18.1 ± 3.36	0.2 ± 0.02	0 ± 0	148.8 ± 28.76	5.5 ± 1.01	0 ± 0	10.4 ± 0.77
		Control	96.5 ± 14.6	442.4 ± 57.9	2.6 ± 0.24	21.3 ± 2.81	0.2 ± 0.01	0 ± 0	174.5 ± 19.54	6.7 ± 1.2	0.6 ± 0.06	12.7 ± 2.01

Values for each compound within each time point that are followed by the same letter are not significantly different (P<0.05) *Data is log transformed.