Chitosan application improves resistance to *Fusarium circinatum* in *Pinus patula*

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***Highlights**

- Induced resistance against pitch canker was investigated in *Pinus patula* seedlings.
- Ten chemical and biologically-derived inducers were tested.
- Chitosan (10 mg/ml) was effective in reducing and delaying disease symptoms.
- Treated seedlings showed an up-regulation of *phenylalanine ammonia lyase* (*PAL*).
- The latter result suggests the onset of induced resistance in *P. patula.*

Abstract

Fusarium circinatum is the causal agent for the disease, pitch canker, in *Pinus patula*. Commercial forestry incurs large economic losses from the pathogen, primarily as a result of post-planting mortality resulting in increased re-establishment costs. One means of enhancing defence is through pretreatment of seedlings with chemicals or biologically derived compounds that stimulate defence responses; a process collectively known as induced resistance. We compared the efficiency of ten inducers in improving defence against *F. circinatum* in *P. patula* seedlings. Chitosan (10 mg/ml) was effective in reducing and delaying disease symptoms of pitch canker in seedlings. Under both nursery and greenhouse conditions, chitosan application resulted in reduced lesion lengths in treated plants compared to non-treated plants over a period of six weeks ($p<0.05$, Kruskal-Wallis). Reverse transcription-quantitative PCR expression analysis revealed that the reduction in lesion size in treated seedlings was accompanied by a four-fold increase in transcript abundance of the *phenylalanine ammonia lyase* transcript, which encodes an enzyme involved in the first committed step of the phenylpropanoid pathway. We suggest that the application of chitosan as part of an integrated management strategy, be further investigated for an effective approach to induce resistance in *P. patula* seedlings against *F. circinatum*.

1. Introduction

The fungal pathogen, *Fusarium circinatum*, affects a range of commercially important hosts worldwide (reviewed by Wingfield *et al*., 2008). The pathogen causes pitch canker that is characterized by the development of large resinous cankers at the sites of

infection in natural stands and plantations of susceptible pine tree species (Wingfield *et al.*, 2008). In commercial nursery seedlings, the major symptoms of infection include wilting, chlorosis, discoloration and damping of seedlings leading to seedling and tree death (reviewed by Mitchell *et al*., 2011). In South Africa, the pathogen is also associated with significant reductions in post-planting establishment of *Pinus patula* seedlings in the field, which is thought to be as a result of seedling contamination in the nursery (Mitchell *et al.*, 2011).

Current disease management strategies are based on an integrated approach focusing on the use of appropriate nursery hygiene practices and the use of planting stock that are more tolerant to the pathogen (Mitchell *et al*., 2011). An alternative disease management strategy exploits the phenomenon of induced resistance in plants, which is potentially useful in improving resistance against a subsequent challenge by a pathogen (Eyles *et al.*, 2009; Vallad and Goodman, 2004). The use of induced resistance has been explored extensively in agriculturally important crop plants such as maize, wheat and tomatoes (reviewed in Sticher *et al.*, 1997; Vallad and Goodman, 2004).

There are three types of induced resistance: (i) systemic acquired resistance (SAR) that is activated by necrotic lesion-inducing pathogens causing a hypersensitive response (HR; Durrant and Dong, 2004) (ii) systemic induced resistance (SIR) that is induced by biotic or abiotic elicitors not eliciting an HR and (iii) induced systemic resistance (ISR) that is activated by the exposure of roots to specific strains of plant growth-promoting rhizobacteria and fungi (Van Loon, 2007; Vinale *et al.*, 2008; Eyles *et al*., 2009). The

defence process induced after application of a biological or chemical elicitor is a highly co-ordinated and integrated response which involves anatomical modifications (e.g. lignification) and production of chemicals (e.g. the production of terpenoids) and proteins (e.g. pathogenesis related [PR] proteins) with antimicrobial activity. SAR is salicylic acid-dependent and associated with the accumulation of PR proteins. In contrast, ISR is dependent on the synthesis of ethylene and jasmonic acid, independent of salicylic acid, and not associated with the accumulation of PR proteins (Vallad and Goodman, 2004). SIR is closely linked to ISR as it also involves the synthesis of jasmonic acid, ethylene and the activation of systemic proteinase inhibitors and wound response proteins (Gurr and Rushton, 2005).

Several biological elicitors have been demonstrated to be effective for inducing the resistance to a range of pathogens in *Pinus* and other coniferous species (reviewed by Eyles *et al.*, 2009). For example, the use of plant growth-promoting bacteria such as *Bacillus pumilus* and *Serratia marcescens* to pretreat seeds significantly increased the resistance of *Pinus taeda* seedlings to fusiform rust caused by *Cronartium quercuum* f.sp. *fusiforme* (Enebak and Carey, 2000). In *Pinus radiata*, foliar applications of chemical compounds such as salicylic acid and its derivatives have been shown to enhance resistance against *Diplodia pinea* (Reglinski *et al.*, 1998), while fungal-derived compounds such as chitin or chitosan improved resistance to *F. circinatum* (Reglinski *et al.*, 2004). Pre-exposure of *Pinus* species to fungal pathogens also enhanced their resistance to later infections. Inoculation of *Pinus nigra*, for example, significantly reduced the lesion lengths that were produced after subsequent inoculations with

Diplodia pinea (Blodgett *et al.*, 2007; Wallis *et al.*, 2008). A similar effect was shown for *P. radiata* that were artificially pretreated with, or naturally pre-exposed to *F. circinatum* (Bonello *et al.*, 2001). In fact, the natural occurrence of induced resistance in *P. radiata* is thought to be one of the factors responsible for the observed remission of pitch canker in areas of California where the disease was first observed (Gordon *et al.*, 2011).

The onset of induced resistance can be assessed at the molecular level by analyzing the expression levels of several key diagnostic genes. Examples of such diagnostic genes follow. The *flavin dependent monooxygenase 1 (FMO1)* and *thaumatin* (*PR-5)* genes are associated with SAR. The *lipoxygenase (LOX), chitinase (PR-3)* and *plant defensin (PDF1.2)* genes are associated with methyl jasmonate (MeJA) and ethylene (ET; Brodersen *et al.*, 2006; Bruinsma *et al.*, 2010; Kawamura *et al.*, 2009). The *1-deoxy-D-xylulose-5-phosphate synthase 1 (DXS1)* gene is associated with the production of terpenoids (Phillips *et al.*, 2007; Zulak and Bohlmann, 2010). The *phenylalanine ammonia lyase (PAL)* gene is associated with the phenylpropanoid pathway (Vogt, 2010; Yang *et al.*, 2011) which may lead to the production of secondary metabolites and cell wall lignification as a branch of defence.

The overall objective of this study was to evaluate the use of induced resistance in *P. patula* seedlings in protection against *F. circinatum*. The first aim was to select and evaluate appropriate elicitors of this process by screening a panel of chemical- and biologically-derived treatments for their efficacy in inducing resistance against *F. circinatum* in *P. patula* seedlings. The second aim of this study was to determine

whether the observed induced resistance in *P. patula* could be correlated with increased expression levels of known defence response genes. The findings of this study will contribute to the improvement of current *F. circinatum* disease management strategies and also towards future studies aimed at elucidating the molecular basis of induced resistance in *P. patula* and other *Pinus* species.

2. Materials and methods

2.1 Plant materials

Pinus patula seedlings used in this study originated from an orchard mix of seeds representing 12 open pollinated families obtained from Mondi South Africa. Seeds were sown into industry standard Unigrow trays comprising 128 plastic inserts filled with a pine bark soil mix.

2.2 Selection of elicitor concentrations

To investigate the possible toxic effect of high concentrations of inducers on the seedlings, suitable concentrations for the active ingredients (a.i) in each of the ten chemical or biologically-derived compounds were tested. Suspensions of 100 µM, 250 µM and 500 µM MeJA (SIGMA-Aldrich, USA, Brownfield *et al.*, 2008); 0.05 g/L, 0.1 g/L and 0.3 g/L Bion® (Syngenta Crop Protection, USA, Dietrich *et al.*, 2004); 1.2 mM, 2 mM and 5 mM sodium salicylate (as a source of SA treatment; Riedel-de Haën, USA, Yao and Tian, 2005) and 10 g/L potassium phosphate monobasic (1% active ingredient, SIGMA-Aldrich, Reuveni *et al.*, 2000) were dissolved in water with 0.1% (v/v) tween®20 (SIGMA-Aldrich), as a surfactant to allow longer adhesion, and 0.1% (v/v) ethanol

(Brownfield *et al.*, 2008). The commercial products Kannar® and Messenger® were dissolved in water to a concentration of 2 ml/L for Kannar® (Kannar Earth Sciences, South Africa) and 65 mg a.i/L for Messenger® (Insect Science, South Africa) as suggested by the manufacturer.

Biologically-derived treatments were prepared as follows. *Pseudomonas fluorescens* was grown for 48 h on Luria-Bertani (LB) plates. LB plates were composed of 1% tryptone powder (Merck, SA), 0.5% yeast extract (Oxoid chemicals, England), 1% NaCl (Merck) and 1.5% agar bacteriological (Merck). The bacteria was subsequently scraped from the plates, added to 500 ml LB broth and incubated overnight at 200 rpm at 26°C. Bacteria were diluted within 2.5 L water (Lemanceau *et al.*, 1992). A nonpathogenic *Fusarium oxysporum FO47* spore suspension was obtained by growing the fungus on half-strength PDA plates as described by Belgrove *et al.* (2011) .The plates were then flooded with 2 ml of 15% (v/v) glycerol to obtain the mycelium. The concentration was determined using a haemocytometer and $5x10^5$ spores/plant were applied to the seedlings as a soil drench (Belgrove *et al.*, 2011). The crude bacterial elicitor was prepared from *Ralstonia solanacearum* as described previously for *Pseudomonas syringae* (Felix *et al.*,1999). The bacteria were cultured in 50 ml tubes. To pellet the bacteria, the tubes were centrifuged at 1000 rpm for 10 min at 4°C. The bacteria was resuspended in 10% of the original volume and boiled for 10 min at 95°C, after which it was centrifuged for 10 min (1000 rpm) to collect debris. The supernatant was then diluted in a 1:9, bacterial elicitor to water ratio and used for spraying. Chitin, derived

from crab shells (SIGMA-Aldrich), was prepared in water to concentrations of 1 mg/ml, 10 mg/ml and 100 mg/ml (Zhang *et al.*, 2002).

Each of the treatments was sprayed onto a small set of plants in the nursery to run-off with a small volume hand mister. Water was applied to the control plants. After 24 h the plants were examined for the development of any necrotic symptoms. This was performed in order to determine the highest concentration that could be applied that had the least toxic effect on the plant.

2.3 Evaluation of elicitors for induced resistance in *P. patula* seedlings

Two separate and consecutive elicitor screening experiments were performed. The first experiment was conducted in a section of a commercial nursery (Top Crop Nursery, Pietermaritzburg, South Africa), which is not used for commercial pine seedling production and that did not receive standard hygiene practices. The second experiment was conducted in a controlled greenhouse (at the FABI disease free facility, University of Pretoria, South Africa) under *F. circinatum* associated disease-free conditions.

The screening experiment in the nursery utilized a set of four-month old seedlings (440 plants per treatment). These plants were respectively treated with, Bion® (0.3 g/L), Messenger® (65 mg a.i./L), Chitin (1 mg/ml), MeJA (500 µM), *Fusarium oxysporum FO47* ($5x10^5$ spores/plant), *P. fluorescens* (6 ml), SA (2 mM), Kannar® (1 in 500 ml), *Ralstonia solanacearum* (crude bacterial elicitor) and potassium phosphate monobasic (10 g/L). Two months after the first application, a booster application of the same concentration was applied. A set of 440 plants received no inducer application. Instead

they were sprayed with water and served as the negative control. A subset of healthy seedlings was artificially inoculated with *F. circinatum* (see below) a week after the booster application. This trial was arranged in a randomized complete block design comprising 11 treatments (ten elicitors and one control; 80 seedlings per treatment) of 16 plants per plot, replicated five times. The plants were spread across trays where each tray contained four plots and a row was left open between plots to prevent any cross contamination.

The screening experiment under greenhouse conditions included a set of 784 four-month old *P. patula* seedlings. These seedlings were evenly distributed into groups for the application of MeJA, Messenger® and chitosan (SIGMA-Aldrich, Inc.), and three control treatments. Chitosan was prepared according to the protocol outlined by Reglinski *et al.* (2004) and applied at two different concentrations (1 mg/ml and 10 mg/ml) that had previously shown a positive effect (Reglinski *et al.*, 2004; Zhang and Punja, 1994). Each of the three inducers had a separate control with 112 seedlings. The control treatments were water for Messenger® and chitosan and a mixture of water, 0.1% tween®20 and 0.1% ethanol for MeJA. The treatments were applied to the plants as described before, and the second application (booster) was administered when the plants were six months old using the same treatment regime. A week after the booster application was applied the plants were subjected to artificial inoculation with *F. circinatum* (see below). This trial was arranged in a randomized complete block design comprising seven treatments of 16 plants per plot, replicated eight times.

In both experiments, the efficiency of the various chemical and biologically-derived treatments to induce resistance in *P. patula* to *F. circinatum* was investigated by scoring symptom development over an eight-week period. The appearance of the plants was rated according to the severity of the disease symptoms on a scale of 1-3, where 1 = healthy, 2 = wilting or yellow-red needles, and 3 = needle discolouration or dead. For each plant the percentage Livestem was also calculated from seedlings height and lesion length (Hodge and Dvorak, 2000).

The statistical software package Analyse-it® (Analyse-it Software, Ltd., Leeds, UK) was used to perform Shapiro-Wilk's tests for normality. To determine significance of effect differences between the various treatments and their controls, the pairwise comparison Kruskal-Wallis test was performed ($p<0.05$).

2.4 Artificial inoculation of pine seedlings with *F. circinatum* to evaluate elicitors

Inoculum for the artificial inoculations was prepared from three isolates of *F. circinatum* (FCC3577, FCC3578 and FCC3579) that were obtained from the Tree Protection Co-operative Programme (TPCP, FABI, University of Pretoria, South Africa). Each isolate was individually grown on half-strength PDA plates as described by Porter *et al*. (2009) at 25°C under fluorescent light with 12 h light/dark periods. From 10-day old cultures of each isolate, spore suspensions in 15% (v/v) glycerol were prepared and quantified using a haemocytometer. These were then combined in equal ratios to a final concentration of $5x10^4$ spores/ml. Following the removal of the apical bud from each seedling, approximately 10 µl of the spore suspension was placed onto the wound.

2.5 Identification of differentially regulated defence genes in *P. patula* in response to chitosan application

The transcript levels of *P. patula* orthologs of known defence genes were investigated 24 h after the booster treatment with chitosan. These genes were *flavin-dependent monooxygenase 1* (*FMO1,* Mishina and Zeier, 2006) and *chitinase* (*PR-3,* Shoresh *et al.*, 2010) representing the SAR and ISR pathways, respectively. The *phenylalanine ammonia lyase* (*PAL*) gene was selected as a marker for the phenylpropanoid pathway (Wang *et al.*, 2008) and *1-deoxy-D-xylulose-5-phosphate synthase 1* gene (*DXS1*) for terpene defence (Estévez *et al.*, 2001).

Gene sequences for *FMO1*, *PR-3*, *PAL, ADP-ribosylation factor (ARF), proteasome subunit (26S)* in *Arabidopsis* and *DXS1* in *Picea abies* were obtained from The *Arabidopsis* Information Resource (TAIR, http://arabidopsis.org) and GenBank (www.ncbi.nlm.nih.gov), respectively. The gene accession numbers are indicated in Table 1. These genes were used as query sequences in TBLASTX searches against the ConiferGDB database *(*www.conifergdb.org) containing *Pinus taeda* expressed sequence tags (ESTs). *Pinus* ESTs with E-values smaller than 10^{-50} were then analysed using GeneMark (http://exon.gatech.edu/eukhmm.cgi) and GenScan (http://genes.mit.edu/GENSCAN.html) to predict the CDS and amino acid sequences. Overlapping ESTs were assembled into contigs where possible. Proteins were aligned using ClustalW (www-bimas.cit.nih.gov/clustalw/clustalw.html) and MAFFT (http://mafft.cbrc.jp/alignment/server). Neighbour - joining trees and maximum likelihood

trees were constructed for each gene. Phylogenetic analysis was performed using MEGA4 (www.megasoftware.net*)* and PhyML (Guindon and Gascuel, 2003*).* Amino acid sequences from members of multi-gene families in *Arabidopsis* were included to differentiate between orthologous *P. taeda* gene family members and the *P. taeda* sequence with the closest relationship to the original query sequence (Table 1) was selected as the putative ortholog. Reciprocal TBLASTN analysis using the putative *P. taeda* ortholog as a query against the *Arabidopsis* database returned the *Arabidopsis* target sequence as the best hit (E-value<10^{-50})*.*

Primer Designer 4 v. 4.20 (Sci Ed Central, Cary, North Carolina, USA) was used to design primers for amplification of 100-350 bp regions of the selected orthologs. To validate the specificity of these primers, amplicons were resolved using agarose gel electrophoresis and the DNA sequences were determined from the purified PCR products. The sequences were analysed using TBLASTX comparisons against expressed sequences in TAIR, NCBI and ConiferGDB databases.

Expression of *flavin-dependent monooxygenase 1 (PpaFMO1)*, *chitinase* (*PpaPR-3)*, *phenylalanine ammonia lyase* (*PpaPAL)* and *1-deoxy-D-xylulose 5-phosphate synthase 1* (*PpaDXS1*) in chitosan-treated *P. patula* was quantified using reverse transcription-quantitative PCR (RT-qPCR). Tissue was harvested from shoots of seedlings 24 h after the booster treatment with 10 mg/ml chitosan and from control seedlings. Total RNA was extracted from 1-3 g of tissue according to Chang *et al.* (1993). RNA quantity and quality were determined using a Nanodrop® ND-100 Spectrophotometer (Nanodrop

Technologies, USA) and Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). After treatment with RNase-free DNaseI (Qiagen Inc, Valencia, CA) to remove any possible DNA contamination, first-strand cDNA was synthesized using Promega's ImProm-II™ Reverse Transcription System (Promega, Wisconsin, USA) from 1 µg RNA.

The qPCR adhered to MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Bustin *et al.*, 2009). Each 11 µl qPCR reaction consisted of LightCycler® 480 SYBR Green I Master mix (Roche, Mannheim, Germany), 10 nM of each primer and 1 µl of a 1:20 dilution of cDNA template. The PCR was executed on the LightCycler® 480 Real-Time PCR System (Roche Diagnostics, GmbH, Basel, Switzerland) with an initial preincubation step of 95°C for 5 min. This was followed by 45 cycles of amplification at 95°C for 10 sec, 64°C for 10 sec and 72°C for 15 sec with fluorescence detected at each cycle. Melting curve analysis was performed by denaturing the DNA at 95°C for 5 sec, followed by a cooling step to below the annealing temperature of the target DNA (65°C for 1min). Samples were slowly reheated to 95°C and sample fluorescence was measured at each degree increment. Each biological sample was amplified in triplicate for technical repeatability. Following evaluation of various housekeeping genes for stable expression (M = 0.468, CV = 0.162), *ADP-ribosylation factor* (*PpaARF1*) and *proteosome subunit* (*Ppa26S*) were selected as references genes. All primer sequences are indicated in Table 1. Crossing points were calculated by determining the absolute quantification /2nd derivate Max and data was imported into qBASE*plus* v1.0 (www.qbaseplus.com Biogazelle, Ghent, Belgium) where the relative quantification and normalization (Hellemans *et al.*, 2007)

was performed. Significance was assessed using a one-tailed Student's t-test (p<0.05) contrasting induced and uninduced seedlings and graphs were prepared in Microsoft Office Excel 2007.

Table 1. *P. patula* primer sequences designed for specific defence gene targets

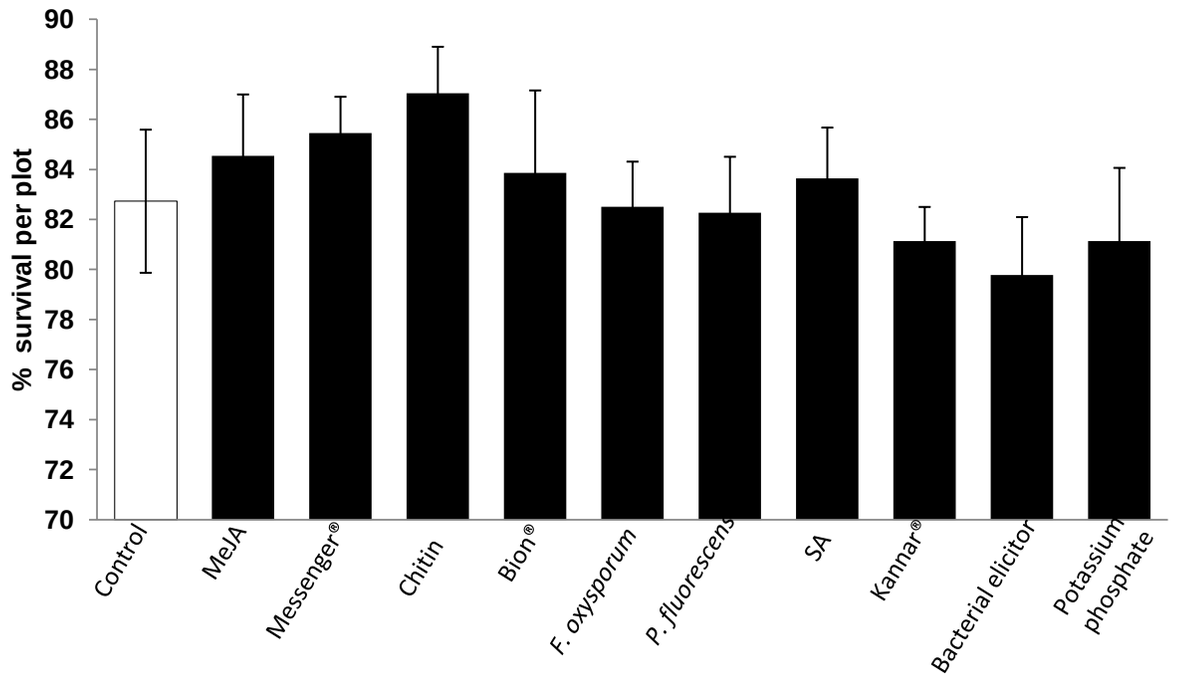
Target gene	Name	Forward primer 5'-3'	Reverse primer 5'-3'
Arabidopsis At1g19250	*PpaFMO*	TGGCCATCCTTGTACAGTG	GATAGTACGCCGTGCAGAA
Picea abies ABS50518	*PpaDXS1*	CAGTTGCAGATGCCAGATTC	TACTGATGCCGCGATATGAG
Arabidopsis At2g37040	*PpaPAL*	TCAAGAACGCAGAAGGTGAG	GACTAACTTGCGGTTCAAGC
Arabidopsis At1g02360	*PpaPR-3*	ATGGAATGGTGACGGACATC	CCAGCATGTTGCAGTATCTC
Arabidopsis At3g04720	*PpaPR-4*	GCAGCAAGCGTCCAATGT	ATGCGCCAGTGTCTCTGT
Arabidopsis At1g09100	*Ppa26s*	GGCCTGACACTCTTGATCCT	CAGTGCAGACACTCCGAATG
Arabidopsis At1g23490	*PpaARF1*	GATCTCTAACAGGCGGTCAA	TCTCCATAGTGGACGGATCT

3. Results

3.1 Evaluation of inducers for induced resistance in *P. patula* seedlings

The efficacy of a panel of chemical and biological inducers to improve resistance against pitch canker disease was assessed on seedlings in a nursery environment with inherently high inoculum levels of *F. circinatum* as well as under artificial inoculum conditions. Several concentrations of inducers were tested on a sub-set of *P. patula* seedlings to determine the optimal concentration for application. The highest inducer concentrations were selected for further application since in each case, the highest inducer concentrations did not result in toxicity and no evidence of chlorosis was observed (results not shown).

FIG 1. Effectiveness of chemical and biological inducers in enhancing survival of P. patula seedlings compared to untreated plants under nursery conditions. The number of dead plants was counted in each replicate and the average percentage of plant survival per replicate was calculated for 440 plants per treatment. Error bars represent standard deviation.



Inducers were applied to *P. patula* seedlings at four months old. The plants received a booster application at six months old and were maintained in a private nursery. Most of the seedlings displayed wilting symptoms typical of *F. circinatum* infection (tip die-back, reddish brown discoloration of needle; Mitchell *et al.*, 2011). A number of plants were randomly selected for testing and confirmed to be infected by *F. circinatum* (TPCP Diagnostic Clinic, FABI, University of Pretoria). The application of Bion®, Messenger®, MeJA, Chitin and SA appeared to improve the survival of plants compared to the control plants (FIG 1), however this was not statistically significant as revealed by the Kruskal-Wallis statistical test ($p<0.05$).

Of the surviving plants, eighty plants per inducer were artificially inoculated with *F. circinatum* and the percentage Livestem assessed post-inoculation. Comparisons of the percentage Livestem revealed that seedlings treated with chitin had significantly reduced lesion lengths ($p<0.01$, Kruskal-Wallis test), with more than an average of 80% of the stem remaining healthy (FIG 2), compared to 68% for control seedlings ($p<0.004$). The treatment with Bion® ($p<0.03$), Messenger® ($p<0.05$) and MeJA ($p<0.05$) also significantly reduced disease severity caused by *F. circinatum* on the seedlings as indicated by higher percentage Livestem compared to the control plants (FIG 2). Interestingly, treatments with Kannar® ($p<0.005$) and *P. fluorescens* ($p<0.03$) appeared to have had the opposite effect and caused a reduction in the percentage Livestem compared to the control plants (FIG 2).

FIG 2. Disease symptoms in P. patula seedlings, six weeks post artificial inoculation with F. circinatum after treatment with the various inducers. Eighty plants per treatment were evaluated to determine the percentage Livestem. Error bars represent standard deviation. The ** indicates p<0.01, while * indicates p<0.05 between treated and control plants (Kruskal-Wallis test).

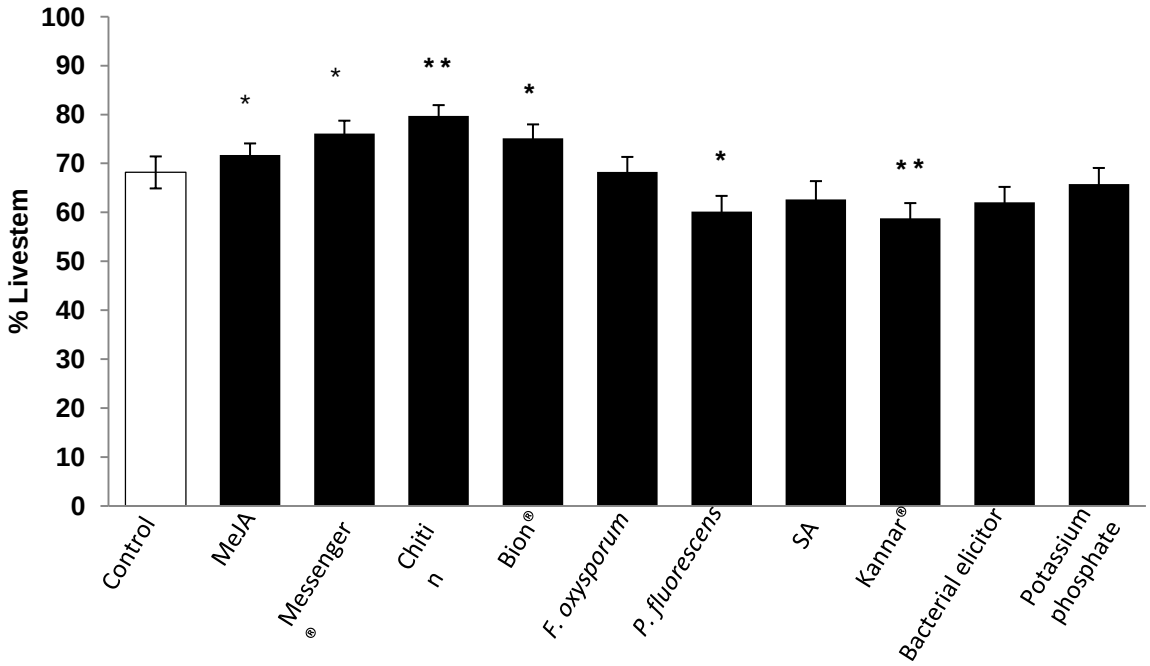
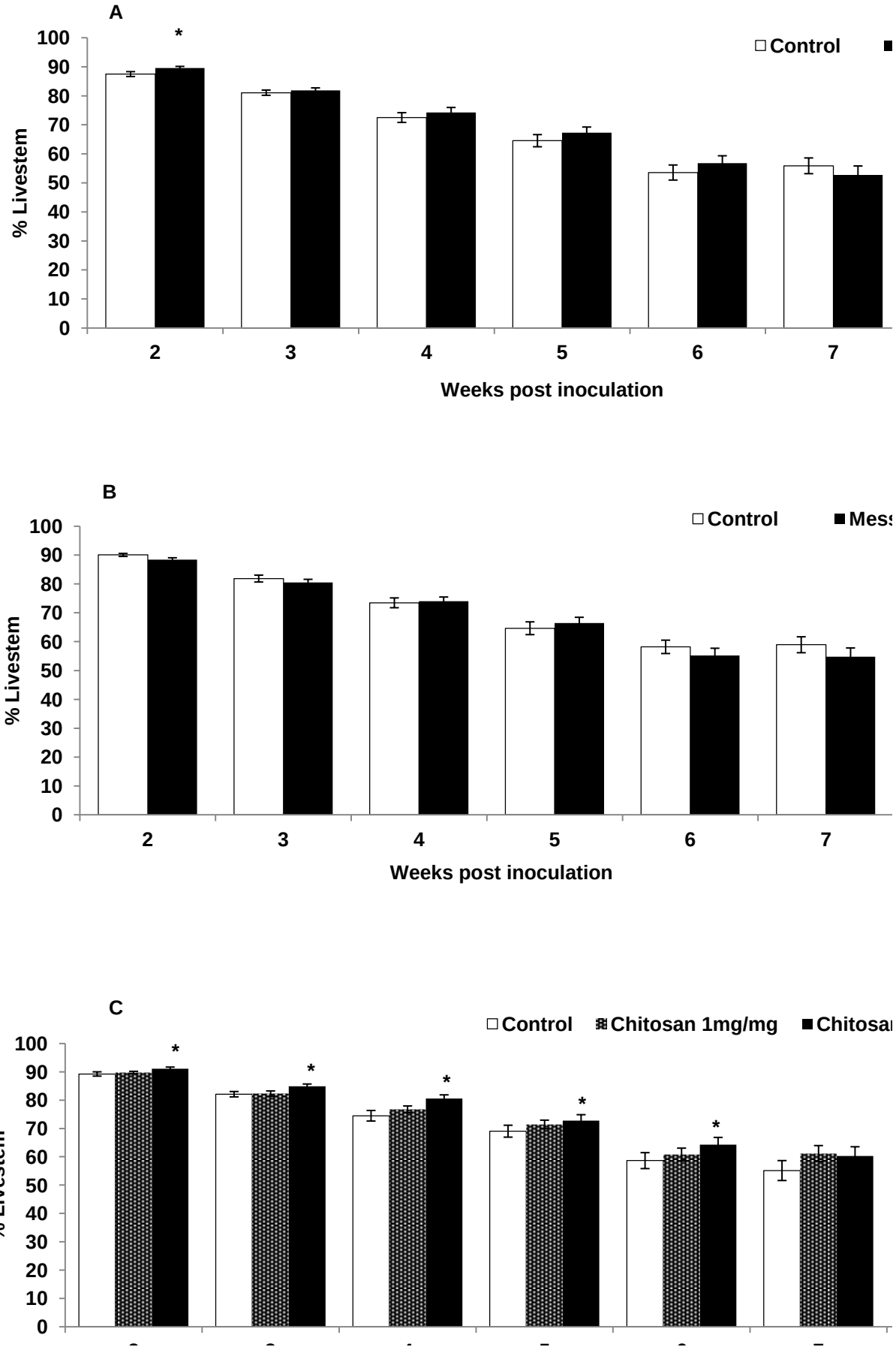


FIG 3. The effect of A) MeJA, B) Messenger® and C) Chitosan treatment on disease progression in P. patula seedlings during an eight week period post inoculation with F. circinatum. A set of 112 plants per treatment were assessed to determine the percentage Livestem. The controls for both MeJA and Chitosan were a mixture of water, ethanol and tween®20 while the control for the Messenger® treatment contained only water. Error bars represent standard deviation. The * indicates p<0.05 (Kruskal-Wallis test) between the induced and control plants.

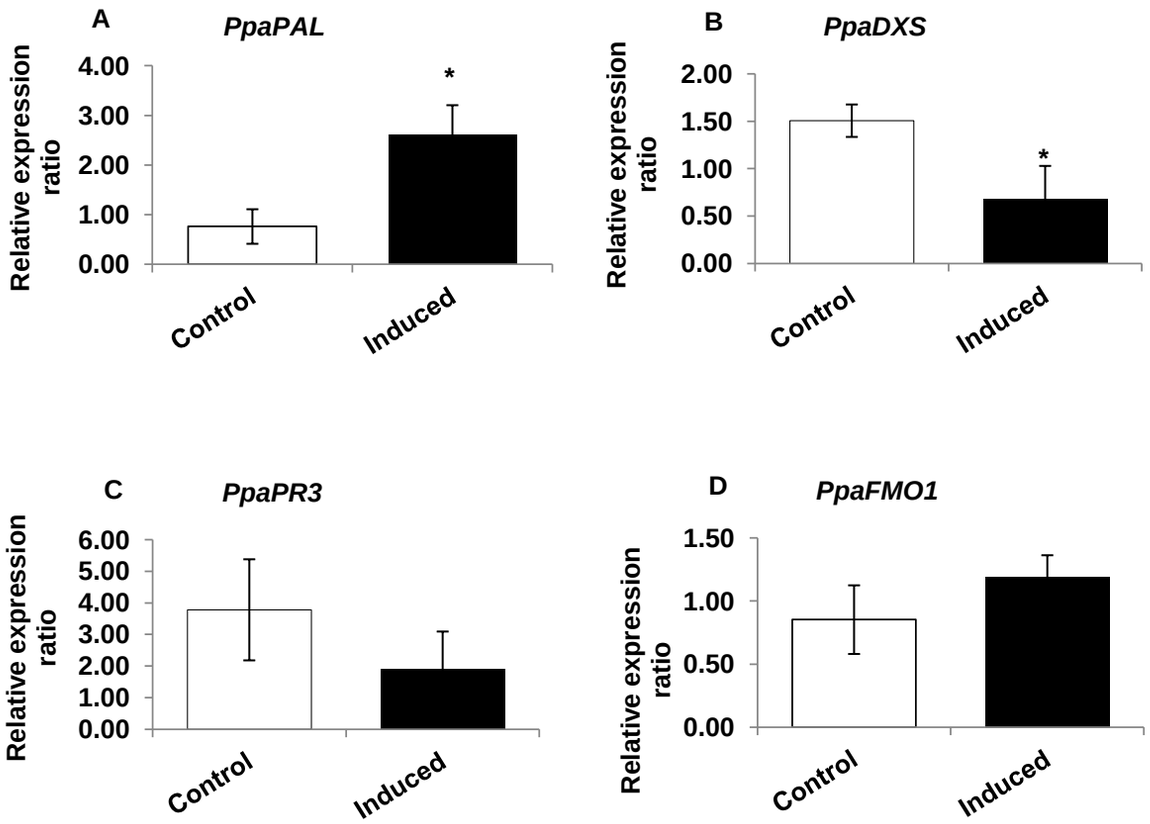


The effectiveness of deacetylated chitin or chitosan, Messenger® and MeJA to induce resistance to *F. circinatum* was assessed under greenhouse conditions. During the eight-week period after artificial inoculation with *F. circinatum,* the percentage Livestem in the MeJA treatment (week two post inoculation) and the 10 mg/ml chitosan treatment (from week two to six post inoculation) was significantly higher ($p<0.05$, Kruskal-Wallis) than those for their respective control treatments (FIG 3). The percentage Livestem difference between the chitosan treated and the control seedlings was small (~ 6%). The percentage Livestem for the remainder of the time points for MeJA and chitosan treatments did not differ significantly from the respective control treatments. The same was true for all time points for the Messenger® treated plants (FIG 3).

3.2 Differential regulation of defence genes in *P. patula*

In order to relate the reduction of lesion lengths in chitosan treated seedlings to the activation of induced resistance at the molecular level, the expression levels of known defence-related genes *PpaFMO1*, *PpaPR-3*, *PpaPAL* and *PpaDXS1* were assessed. By making use of qPCR analysis of the transcript abundance levels of these genes after the booster application of 10 mg/ml chitosan, significant differential expression ($p<0.05$, Student's t-test) was detected for *PpaPAL* and *PpaDXS1* only (FIG 4). In the *P. patula* plants treated with chitosan the expression level of *PAL* was four times higher than in the control plants not treated with chitosan ($p<0.05$, Student's t-test, FIG 4A). The *PpaDXS1* gene had significantly lower expression in the seedlings treated with chitosan compared to the control ($p<0.05$, Student's t-test).

FIG 4. Relative transcript abundance of putative defence genes in P. patula in response to chitosan application. The white bars represent transcript abundance in the control P. patula seedlings and black bars represent the transcript abundance in P. patula seedlings treated with 10mg/ml chitosan for A) PpaPAL, B) PpaDXS, C) PpaPR3 and D) PpaFMO1. The error bars represent the biological standard error (n=3). The *indicates p<0.05, Student's t-test.



4. Discussion

This study aimed to investigate the effectiveness of various chemical and biologically-derived inducers in reducing disease symptoms caused by *F. circinatum* on *P. patula* and to subsequently determine the activation of induced resistance in *P. patula* at the molecular level. Two important findings have emerged from our observations i.e. (i) chitosan treatment of *P. patula* seedlings resulted in reduced *F. circinatum* disease symptoms and (ii) the application of chitosan resulted in enhanced expression of *PpaPAL* in *P. patula*, suggesting the onset of induced resistance.

4.1 Identification of a promising resistance inducer

Of all the chemically and biologically-derived inducers tested under nursery and greenhouse conditions, chitosan treatment at 10 mg/ml appeared to be a promising application to reduce the severity of subsequent *F. circinatum* infection (FIG 2). In trials conducted on *P. radiata,* it was suggested that chitosan application had caused the activation of systemic resistance (Reglinski *et al.*, 2004). Chitin or chitosan represent major components of the fungal cell wall and mimic the activation of local and systemic defence signaling cascades. Chitosan application has been shown to (i) activate reactive oxygen species (Dongmi *et al.*, 1999), (ii) induce direct chemical defence by inducing phytoalexin synthesis (Conrath *et al.*, 1989; Hadwiger and Beckman, 1980; Köhle *et al.*, 1984), and (iii) induce physical defence in the form of cell wall lignification and callose deposition (Conrath *et al.*, 1989; Grosskopf *et al.*, 1991; Pearce and Ride, 1982). Chitosan has been proven to have inhibitory effects on hyphal growth of fungi (Aranaz *et al.*, 2009) and was recently demonstrated to be effective against *Botrytis*

cinerea in *Arabidopsis* (Povero *et al.*, 2011), making the polymer an attractive foliar treatment (El Hadrami *et al.*, 2010).

Other inducer treatments which showed some promise as resistance inducers included Bion®, Messenger®, Chitin, MeJA and SA applications which appeared to improve survival of the seedlings compared to controls at four months; however these observations were not statistically significant (FIG 1). Artificial inoculations a week after booster application revealed that Chitin, Messenger® and Bion® treated plants had significantly smaller lesion sizes compared to the control plants suggesting that these treatments had provided a level of protection against the pathogen (FIG 2) albeit for a limited period. These applications have been successfully applied in rice where chitin treatment demonstrated effectiveness against the fungus *Magnaporthe oryzae* (Kishimoto *et al.*, 2010) and in tomato, cucumber and wheat where Messenger® was used against the soil-borne pathogen *Fusarium* spp. (Jones, 2001), while Bion® was effective in *P. radiata* against *Phytophthora cinnamomi* (Ali *et al.*, 2000). It is recommended that the dose, frequency and timing of these applications have to be optimized to be effective treatments to enhance resistance in *P. patula* against *F. circinatum*.

In the first set of inoculation experiments, treatment with Messenger® appeared to have improved defence against *F. circinatum* however this trend did not prevail in the second trial. It is suspected that this result is due to the slightly higher temperature conditions during the second set of artificial inoculations. The higher temperature may be

responsible for an increase in pathogen virulence, a phenomenon which has been previously observed in *P. radiata* inoculated with *Fusarium subglutanins* (McDonald, 1994). The increased virulence of *F. circinatum* may have abolished the marginal resistance that may have been mediated by Messenger® treatment.

Unexpectedly, two inducers (Kannar® and *P. fluorescens*) appeared to enhance disease severity (FIG 2). This phenomenon could be due to the activation of the inappropriate defence pathway. For example, it has been demonstrated that JA/ET and SA defence responses are tailored to the type of pathogen, the former defence signaling pathway being more effective against necrotrophs and the latter against biotrophs (Glazebrook, 2005).

F. circinatum has been established in South Africa since the late 1990s and commercial nurseries have accumulated high concentrations of the fungus (Mitchell *et al.*, 2011; Viljoen *et al.*, 1994). The evaluation of the inducers in the nursery environment reflected the commercial conditions under which *P. patula* seedlings would be grown. The presence of the pathogen may have caused an early induction of systemic resistance in some of the seedlings, masking the actual effect of the applied inducer. Literature has shown that *P. radiata* is able to react to repeated infections with *F. circinatum* in a behavior described as induced resistance, observed by the presence of smaller lesions upon subsequent infection (Bonello *et al.*, 2001). In the greenhouse trial, where seedlings were raised in a disease-free environment, any response could be directly related to the effect of the inducer treatment.

Studies have been conducted to explain the correlation between mortality and lesion length. Gordon *et al.* (1998) showed that resistance is quantitative with a continuous range of variation in lesion length. A seedling with a smaller lesion length is more likely to survive than a seedling sustaining a longer lesion. A comparison between greenhouse trials and field studies on *P. radiata* indicated a correlation between the lesion length seen in the greenhouse and the survival observed in the field (Hodge and Dvorak, 2000; Smith, 2011). This observed correlation between greenhouse and field trial results allows the evaluation of resistance in pine species using artificial techniques as an indicator of field performance.

4.2 Defence responses elicited by chitosan treatment in *P. patula*

Since the application of chitosan appeared to reduce disease symptoms caused by *F. circinatum* in *P. patula*, we explored whether the perceived induced resistance was associated with the differential regulation of known diagnostic defence genes at the molecular level. In the absence of genomic resources for *P. patula*, the RT-qPCR expression analysis provided a platform for studying defence responses in this tree species using primers designed from candidate *P. taeda* orthologs. The availability of the *P. taeda* genome sequence in the near future would improve confidence in predicting candidate orthologs.

Using our current molecular approach, the application of chitosan resulted in four fold induction of the transcript of the key enzyme of the phenylpropanoid pathway. This

pathway has various facets ranging from secondary metabolite production to lignification, both being mediators towards resistance in the plant (Vogt, 2010). Experiments performed on carrots and grapevines revealed an induction in transcript levels of *PAL* after treatment with chitosan. These plants showed increased tolerance when inoculated with a necrotrophic fungus (Ferri *et al.*, 2009; Jayaraj *et al.*, 2009). *PAL* plays a role in the carbon flux that is needed for the phenylpropanoid pathway (Hahlbrock and Scheel, 1989). The products that result from this pathway are flavenoids, isoflavenoids, lignin and phenolics (Osakabe *et al.*, 2009). These products lead to the activation of anatomical defences such as lignification of the cell wall and chemical defences relating to the production of flavenoids. Accumulation of phenolic compounds is associated with both passive and systemic defence and the magnitude of defence is linked to the accumulation of precursors of phenolic acids (Singh *et al.*, 2010).

Expression profiling also revealed differential regulation of *PpaDXS1*. A three-fold down-regulation was observed at 24 h after the booster application of chitosan. This gene, *PpaDXS1* is one of two types of plant *DXS* genes known to play a role in catalysing the first steps of the methyl-erythritol phosphate pathway and is important for products such as pyridoxal, thiamine and terpenes (Phillips *et al.*, 2007). Additionally, DXS promotes the onset of isopentenyl diphosphate and dimethylallyl diphosphate pathways (Kim *et al.*, 2009).

One of the terpenes commonly associated with coniferous trees, is resin. This type of resistance barrier is known to be present in older trees, but is not the line of defence in place in young seedlings. It was hypothesised that chitosan may increase the expression of the resin defence, however the profiling of *PpaDXS1* suggests that the pathway was suppressed. The down-regulation may be ascribed to altered carbon partitioning leading to suppression of some defence pathways such as the methyl-erythritol phosphate pathway in order to accommodate the increased carbon required for the phenylpropanoid pathway. Carbon within the first year of growth is mostly assigned to the growth of the plant instead of the production of resin. After the growth spurt of the first year, drastic growth was observed in duct size and the amount of resin (Hudgins *et al.*, 2006; Wainhouse *et al.*, 2009).

P. abies (Norway spruce) *PaDXS1, PaDXS2A* and *PaDXS2B* transcripts showed differential expression to wounding, chitosan, methyl salicylate and *Ceratocystis polonica* treatment; suggesting distinct functions of the three DXS genes in primary and defensive terpenoid metabolism (Phillips *et al.*, 2007). Protein abundance of the three DXS isoforms PaDXS1, PaDXS2a and PaDXS2b were investigated in *P. abies* after MeJA treatment and high abundance was noted for PaDXS2a and PaDXS2b at 4 and 8 days post treatment in comparison to control tissue (Zulak et al., 2009). The abundance of PaDXS1 was not as high as the other isoforms. Transcript abundance for *PaDXS1* spiked 2 days after MeJA treatment and then declined while transcript abundance of *PaDXS2a* and *PaDXS2b* remained high up to 16 days after MeJA treatment (Zulak et al., 2009).Thus, profiling of *PpaDXS2a* and *PpaDXS2b* should also be conducted on

chitosan treated *P. patula* seedlings at various time-points and stages of development as an indicator of terpenoid defence in *P. patula*.

Flavin-dependent monooxygenase has numerous functions involved in pathogen resistance. The gene is a marker for the SAR pathway in *Arabidopsis* where its local and systemic expression was identified during inoculation with *P. syringae pv. tomato* DC3000 (Mishina and Zeier, 2006). Besides the priming of the SA-dependent defence pathway, *FMO1* also causes the priming of callose deposition and seems to regulate cell death (Ent *et al.*, 2009). Furthermore *FMO1* plays a crucial role in basal defence, as well as for the triggering of TIR-NB-LRR resistance gene-mediated defence (Schlaich, 2007). The expression pattern of *PpaFMO1* in *P. patula* may reflect that SAR is not activated in response to the chitosan treatment.

The chitinase enzymes play a crucial role in hydrolysing chitin, thus it was expected that the application of a deacetylated version of chitin would have an effect on the regulation of *PR-3*. Commonly with fungal attack, *PR-3* is one of the prominent plant genes that will respond (Heil and Bostock, 2002). Chitosan treated carrots and grapevines had enhanced resistance and showed increased levels of PR-3, successfully combating *Alternaria radicina* (Jayaraj *et al.*, 2009). In the current study, the gene showed no significant differential regulation, however it is possible that the candidate ortholog targeted for expression analysis may not represent the functional ortholog. Venter (2004) demonstrated that *P. patula* seedlings challenged with *F. circinatum* showed no detectably induced levels of chitinase transcription. This deficiency was suggested to be

a potential reason as to why *P. patula* is highly susceptible to the fungus (Venter, 2004). It is conceivable that the seedlings revert to alternative pathways, such as the phenylpropanoid pathway, for defence.

In *Arabidopsis* treated plants, chitosan application induced the expression levels of *AtFMO1*, *AtPR-3* and *AtPAL1* to high levels three hours post treatment compared to mock-treated plants ($p<0.03$; Povero *et al.*, 2011). It is possible that the time-point selected in our study (24 h post booster application) was too late to observe changes in these transcripts. Povero *et al*. (2011) showed increased up-regulation of genes associated with the camalexin biosynthetic pathway. Camalexin is one of the major secondary metabolites produced by the tryptophan pathway. The main route for tryptophan, phenylalanine and tyrosine biosynthesis is *via* chorismate intermediates, however phenylalanine can also be synthesized from the intermediate metabolite phenylpyruvate (Tzin and Galili, 2010). The observation of the up-regulation of the camalexin biosynthetic pathway in *Arabidopsis* (Povero *et al.*, 2011) and the phenylpropanoid pathway (as indicated by the up-regulation of *PAL*) in *P. patula* (this study) may reflect distinct routes to the production of secondary metabolites upon chitosan application in the two plant species. Evidence for this hypothesis would have to be derived from expression analysis of transcripts involved in these pathways at various time-points after chitosan treatment in *P. patula*. Interestingly, it was previously discovered that chitosan activates a CERK1-independent signaling pathway however, similar responses to the two polymers (chitin and chitosan) at the molecular level reflect that a convergence downstream of CERK1 is possible (Povero *et al.*, 2011).

In summary, chitosan application appears to hold promise for protection of *P. patula* seedlings against *F. circinatum*. The concentration levels and application strategy remains to be optimized. The up-regulation of the *PpaPAL* transcript in *P. patula* provides a clue as to the possible line of defence important for protection against *F. circinatum.* This observation could be supported by measurement of PAL activity however, the molecular basis of resistance induced by the chitosan treatment on a genome-wide scale would provide further insight into defence mechanisms that exist in *P. patula*, which could be explored for breeding or biotechnology strategies to improve resistance against *F. circinatum* in future.

Acknowledgements

The authors wish to thank Top Crop Nursery, Pietermaritzburg South Africa for the use of their facilities and Mondi Business Paper for providing *Pinus patula* seeds. Funding for this research was provided by the National Research Foundation (NRF) South Africa, Mondi Business Paper and Sappi Forestry. We thank Mrs. Marja Mostert-O'Neill for the critical reading of this manuscript.

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