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Defense-related gene expression in susceptible and tolerant bananas (*Musa* spp.) following inoculation with non-pathogenic *Fusarium oxysporum* endophytes and challenge with *Radopholus similis*

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Abstract

Radopholus similis is a major pest of East African highland cooking bananas (*Musa* spp.) in Uganda. Non-pathogenic *Fusarium oxysporum* endophytes, isolated from bananas in farmers' fields, have shown potential to reduce *R. similis* numbers in tissue culture banana. The mechanism through which endophytes confer resistance to nematodes has previously been demonstrated to involve induced resistance. In this study, the expression of eight defense-related genes in banana was investigated using quantitative real-time reverse transcription PCR. Plants of susceptible (cv. Nabusa, genomic group AAA-EA) and tolerant (cv. Kayinja, genomic group ABB) banana cultivars were inoculated with endophytic *F. oxysporum* strain V5w2. Gene expression levels were analysed following endophyte inoculation and nematode challenge. Endophyte colonization of roots of the tolerant cultivar induced transient expression of *POX* and suppressed expression of *PR-3*, *lectin*, *PAE*, *PAL* and *PIR7A*. *Catalase* and *PR-1* activities were up-regulated in the tolerant cultivar 33 days after endophyte colonization of roots, but their expressions were further up-regulated following nematode challenge. Apart from *POX* and *lectin*, the other genes analysed were not responsive to endophyte colonization or *R. similis* challenge in the susceptible cv. Nabusa. This is the first report of endophyte-induced defense-related gene expression in banana.

Keywords: Banana; *Radopholus similis*; Fungal endophyte; *Fusarium oxysporum*; Gene expression; *Musa*; Quantitative real-time PCR

1. Introduction

Fungal endophytes occur in all plants, and often infect their hosts without causing any observable disease symptoms [1,2]. Apart from grasses, in which they have been intensively studied, endophytes have been isolated from agricultural crops such as banana (*Musa* spp.) [3,4], rice (*Oryza sativa* L.) [5], cocoa (*Theobroma cacoa* L.) [6] and barley (*Hordeum vulgare* L.) [7]. While in the plant, endophytes may be mutualists, commensalists and even parasitic [8,9]. In a mutualistic association between an endophyte and its host, the latter provides nutrients and protection, and aids in transmission to the next generation of hosts for vertically transmitted endophytes. The endophyte in return, is believed to offer increased resistance or tolerance to biotic (pests and diseases) and abiotic (drought and salinity) stresses. The commensalists benefit from the host, but neither harms nor benefits their hosts. Parasites are detrimental to their hosts [10].

The use of pest management practices in Sub-Saharan Africa is severely hampered by economical and environmental constraints. For example, the use of synthetic pesticides is discouraged due to their persistence in soils and their negative effect on the environment and human

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health. These pesticides are also expensive, often unavailable and poorly understood by farmers [11]. For many crops, breeding for host resistance is slow due to the lack of knowledge of resistance mechanisms, resistance markers and genetics of resistance [12]. In banana, durable resistance against major pests such as the burrowing nematode *Radopholus similis* (Cobb) Thorne is yet to be successfully bred into economically important cultivars. Problems associated with the use of traditional pest management practices have led to many crop protectionists turning to control strategies that are naturally occurring and hence environmentally friendly. These include the use of microbial control agents such as entomopathogenic fungi, rhizobacteria and fungal endophytes. Fungal endophytes are particularly attractive for control of pests such as the burrowing nematode because they occur inside the plant where the destructive stages of this pest exist.

Fungal endophytes are beneficial to host plants through various mechanisms, including the production of secondary metabolites, which are used in direct antagonism against pests and diseases [13–17], changes in host physiology, which lead to increased plant growth [18,19], and induction of pest and disease resistance in plants [20–23]. Two types of induced resistance exist. Systemic acquired resistance (SAR) and induced systemic resistance (ISR) can be differentiated on the basis of signal molecules and genes up-regulated following resistance induction, but not their phenotype. SAR is dependent on the salicylic acid pathway and its onset is characterized by expression of genes that encode pathogenesis-related (PR) proteins such as β-1,3-glucanases (PR-1 family), endo-chitinases (PR-3 family) and thaumatin-like proteins (PR-5 family) [24–27]. SAR is widely known to be associated with pathogen attack or in response to the exogenous application of chemicals such as salicylic acid and benzothiadiazole. ISR, conversely, is reported due to root colonization by plant growth-promoting rhizobacteria and its onset is not characterized by accumulation of PR proteins [28–35].

Endophyte-induced defenses in plants are expressed through structural and biochemical mechanisms. Structural mechanisms include the reinforcement of plant cell walls by deposition of newly formed molecules of callose, lignin and phenolic compounds [22,36]. Other physical mechanisms of resistance include the occlusion of colonized vessels by gels, gums and tyloses [37,38]. Physical barriers are formed by the plant to prevent further ingress of the invading organisms [39]. Major biochemical changes following resistance induction include accumulation of secondary metabolites such as phytoalexins [40,41], and production of PR proteins such as β-1,3-glucanases and chitinases [20,21,23]. β-1,3-Glucanase is reported to release elicitors for phytoalexin synthesis [42]. Chitinase and peroxidase enzymes are known to be induced during endophyte colonization [43].

There is evidence that non-pathogenic *F. oxysporum* endophytes can act against *R. similis* through induced resistance. In plant colonization studies, Paparu et al. [44] reported limited *F. oxysporum* endophyte colonization in the root epidermis, speculating that physical barriers prevented further ingress into the root cortex. In screenhouse split-root experiments where the pest and endophyte were inoculated separately onto spatially separated roots, reduced penetration and multiplication of nematodes was observed, demonstrating induced resistance [45]. Also, an increase in production of phenolic compounds was observed in roots inoculated with endophytes and later challenged with *R. similis* [45].

The current study was designed to compare expression of defense-related genes in roots of a susceptible East African Highland banana (EAHB) cultivar (cv. Nabusa, genomic group AAA-EA) and a tolerant banana cultivar (cv. Kayinja, genomic group ABB), following inoculation with endophytic *F. oxysporum* and challenge with *R. similis*. While the biochemical and structural responses of endophyte colonization of plant roots have previously been investigated [45,46], little is known regarding the endophyte-induced resistance pathway and the molecules involved.

2. Materials and methods

2.1. Fungal inoculum and nematode preparation

A non-pathogenic endophytic *F. oxysporum* strain V5w2 was isolated from EAHB plants by Griesbach [3] and is currently stored at the facilities of the International Institute of Tropical Agriculture (IITA) in Kampala, Uganda in soil, on filter paper and in 15% glycerol [47]. Strain V5w2 was chosen because of its demonstrated ability to suppress *R. similis* in screenhouse experiments [45]. From filter paper, the fungus was sub-cultured on half strength potato dextrose agar (PDA) (19 g PDA and 19 g agar l^{-1} distilled water) and a fungal spore suspension prepared 7 days after growth at $\pm 25\,^{\circ}C$. Spore densities were subsequently determined under a light microscope ($100\times$ magnification) using a haemocytometer. Spore concentrations were adjusted to 1.5×10^{6} spores ml^{-1} with sterile distilled water.

Radopholus similis nematodes were cultured on carrot discs according to Speijer and De Waele [48]. The nematodes were originally isolated from banana roots and maintained at 27 °C on sterile carrot discs at IITA, Kampala, Uganda. A nematode suspension was prepared by rinsing nematodes from the carrot discs and from the edge of the Petri dishes using sterile distilled water. A final volume of 110 ml sterile distilled water containing 178 female and juvenile *R. similis* ml^{-1} was obtained.

2.2. Plant material

EAHB cv. Nabusa (highly susceptible to *R. similis*) and cv. Kayinja (tolerant to *R. similis*) were inoculated with the non-pathogenic endophytic *F. oxysporum* strain V5w2. Tissue culture banana plants were propagated using

a standard shoot-tip culture protocol [49]. Four weeks after rooting, plants were removed from the rooting medium and their roots and rhizomes rinsed in tap water. The plants were then replanted to 250 ml plastic cups, with their roots suspended in a nutrient solution [40 ml Micromix® (Magnesium 2%, Sulphur 16%, Manganese 7.5% and Zinc 18%) (Fleuron, Braamfontein, 2017, South Africa), 24 g $Ca(NO_3)_2 \cdot H_2O$ and 36 g Agrasol® (Fleuron) l^{-1} sterile tap water], and their stems secured by the lid of the plastic cups. To enhance root growth, the plants were maintained in a plant growth room set to a photoperiod of 18/6 h light/dark routine, an average temperature of 23 °C and 70% RH for 4 weeks.

2.3. Inoculation of tissue culture plants

Plants were removed from the nutrient solution and their roots washed in distilled water. They were then treated as follows: (1) roots of both cultivars were harvested immediately (0 h) from endophyte-free plants to determine constitutive expression of genes, (2) both cultivars were endophyte-inoculated, and roots harvested 2 days later, (3) both cultivars were endophyte-inoculated, and roots harvested 33 days later, (4) both cultivars were endophyte-inoculated, challenged with *R. similis* at 30 days, and roots harvested 3 days later, and (5) endophyte-free plants of both cultivars were challenged with *R. similis* at 30 days and roots harvested 3 days later. Each treatment had three biological replicates, each consisting of three plants pooled together at harvest.

Plants were inoculated with the endophytic *F. oxysporum* strain by dipping their roots in spore suspensions for 4 h. Non-inoculated plants were dipped in sterile water for the same duration. Plants were then planted in pots (120 mm wide and 90 mm dip) containing sterile soil for the duration of the experiment. Plants were challenged with *R. similis* by excavating the soil at the base of the plant and pipetting 2 ml inoculum (containing approximately 350 nematodes) directly on the roots. The excavated soil was then replaced. Non-challenged plants also had the soil around their roots excavated, but replaced without nematode application. The experiment was conducted in a plant growth room with a photoperiod of 12/12 h light/dark routine and an average temperature of 25 °C, and plants were watered twice weekly. At harvest, root samples were collected for RNA extraction, endophyte re-isolation and nematode extraction. Endophyte re-isolations from roots were undertaken according to Paparu et al. [50] and nematodes extracted using the modified Baermann funnel method [51].

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted from banana roots using the RNeasy Plant Mini Kit (Qiagen, Valencia, USA) according to the manufacturer's instructions. RNA yield was determined by measuring absorbency at 260 nm, using a Nanodrop ND-100 Spectrophotometer (Nanodrop Technologies, Montchanin, USA). One μg RNA was DNAseI treated (Fermentas Life Sciences, Hanover, USA) and first-strand cDNA synthesized by random hexamer priming using Power Script TM Reverse Transcriptase (BD, Biosciences, Belgium) according to the method by Lacomme et al. [52]. The cDNAs were assayed for genomic DNA contamination by PCR using the specific primer set actinF (5′ACCGAAGCCCCTCTTAACCC-3′) and actinR (5′-GTATGGCTGACACCATCACC-3′), and PCR products separated by electrophoresis through a 2% agarose gel containing ethidium bromide.

2.5. Real-time RT-PCR primers

The expression profile of eight banana defense-related genes was analysed in cDNA samples obtained from banana roots (Table 1). Three of these genes, *PR-1*, *catalase* and *pectin acetylesterase* (*PAE*) were previously reported to be up-regulated in a Cavendish selection (genomic group AAA) tolerant to *F. oxysporum* f. sp. *cubense* (*Foc*) (the causal agent of fusarium wilt of banana) [53]. *PAL*, *POX*, *PR-3*, *lectin* and *PIR17* (a *peroxidase*) were found to be up-regulated following *Foc* challenge

Table 1
Primer sequences of defense-related genes studied in roots of banana cultivars susceptible (Nabusa, genomic group AAA-EA) and tolerant (Kayinja, genomic group ABB) to *Radopholus similis* by reverse transcription (RT)-PCR following inoculation with non-pathogenic *Fusarium oxysporum* and challenge with *Radopholus similis*

Target gene[a]	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Product size (bp)
POX[1]	CGGTAGGATCCAAAGAAAGC	TTCAGAGCATCGGATCAAGG	150
PR-3[1]	GTCACCACCAACATCATCAA	CCAGCAAGTCGCAGTACCTC	150
PAL[1]	CCATCGGCAAACTCATGTTC	GTCCAAGCTCGGGTTTCTTC	150
Lectin[1]	CCACGAGGTTTGCATCACTAC	CCCTTCATTCCCACCAGATAC	150
PIR7A[1]	ACCTTCGATCTCCTCCACTTC	GGTCGGTGAGAAGGGTGTT	150
PR-1[2]	TCCGGCCTTATTTCACATTC	GCCATCTTCATCATCTGCAA	126
Catalase[2]	AAGCATCTTGTCGTCGGAGTA	CGCAACATCGACAACTTCTTC	96
PAE[2]	GGCTCTCCTTTCTGGATGTTC	TCAGCAAGGCACTTGACTTTT	105
Musa 25S rRNA[2]	GTAAACGGCGGGAGTCACTA	TCCCTTTGGTCTGTGGTTTC	106

[a] 1—primer sequences previously identified by Forsyth [54] and 2—primer sequences previously identified by Van den Berg et al. [53]. *POX*—peroxidase, *PAL*—phenylalanine ammonia lyase, *PR*—pathogenesis-related and *PAE*—pectin acetylesterase.

of cv. Lady Finger (genomic group AAB) and cv. Cavendish plants previously inoculated with non-pathogenic *F. oxysporum* [54]. Primer sequences for these genes were identified by Van den Berg et al. [53] and Forsyth [54]. An endogenous gene, *Musa* 25S rRNA (AY651067) (5′ACATTGTCAGGTGGGGAGTT3′; 5′CCTTTTGTTCCACACGAGATT3′) [53], was used as a control gene since its expression remains relatively constant. All primers were synthesized by Operon Biotechnologies (Cologne, Germany) (Table 1).

2.6. Gene expression analysis using quantitative real-time RT-PCR

Quantitative real-time reverse transcription (qRT)-PCR was performed using a LightCycler version 1.2 instrument (Roche Diagnostics). The LightCycler FastStart DNA Master^{PLUS} SYBR Green I system (Roche Diagnostics) was used for real-time PCR, using the first strand cDNAs as template. Dilution series and standard curves were performed to examine the linearity of amplification over the dynamic range. A serial dilution (1:10, 1:100 and 1:1000) was performed and used to draw standard curves for all genes. A 10-μl reaction for PCR amplification contained 5 μl FastStart DNA Master^{PLUS} SYBR Green I master mix, 2 μl of forward and reverse primer (10 μM), 1 μl cDNA template and 2 μl PCR grade water (Roche). Control treatments contained water instead of cDNA template. All PCR reactions were performed in triplicate. The cycling conditions were as follows: pre-incubation for 10 min at 95 °C, followed by 55 cycles, each consisting of 10 s denaturing at 95 °C, 10 s annealing at 65 °C, 10 s primer extension at 72 °C, and data acquisition at 95 °C. For PCR amplification of all experimental samples, 1:10 cDNA template dilutions were used.

2.7. Data analyses

Standard regression curves were calculated from amplification data from the serial dilutions [55]. Expression data was normalized using the amplification data for the specific target gene and the endogenous control gene, *Musa* 25S rRNA as previously described [56]. Lightcycler data was subjected to analysis of variance and multiple mean comparisons performed using Tukey's studentized range test. Comparisons between treatments were made using a pooled *t*-test in SAS [57].

3. Results

The expression profiles of defense-related genes produced constitutively (at 0 h) by cv. Kayinja and cv. Nabusa differed significantly, with the exception of *catalase* and *PR-1*, where no significant differences in gene expression levels were found. In the tolerant cv. Kayinja, activities of *PR-3* ($t = 15.59$, $P < 0.0001$), *lectin* ($t = 15.77$, $P < 0.0001$), *PAE* ($t = 3.41$, $P = 0.0024$), *PAL* ($t = 2.30$, $P = 0.029$) and *PIR7A* ($t = 6.27$, $P < 0.0001$) were significantly higher than in cv. Nabusa. The constitutive expression of *POX*, however, was significantly higher in cv. Nabusa compared with the tolerant cv. Kayinja ($t = 3.62$, $P = 0.0014$) (Fig. 1).

When inoculated with the endophyte, gene expression in roots of the tolerant cv. Kayinja and susceptible cv. Nabusa changed dramatically over time. *POX* activity was significantly up-regulated in the tolerant cv. Kayinja 2 days after endophyte inoculation ($t = 6.55$, $P < 0.0001$) and was reduced significantly 33 days after inoculation (Fig. 1A). In the susceptible cv. Nabusa, the expression of *POX* was significantly reduced ($t = 3.15$, $P = 0.0045$) 2 days after endophyte inoculation, but increased again significantly after 33 days. The activities of *PR-3* ($t = 12.47$, $P < 0.0001$), *lectin* ($t = 15.79$, $P < 0.0001$), *PAE* ($t = 3.47$, $P = 0.0021$), *PAL* ($t = 2.70$, $P = 0.012$) and *PIR7A* ($t = 5.35$, $P < 0.0001$) were all significantly reduced in the tolerant cv. Kayinja 2 days after endophyte inoculation (Fig. 1B–F, respectively), but did not change significantly in the susceptible cv. Nabusa. No significant induction of *catalase* (Fig. 1G) and *PR-1* (Fig. 1H) expression occurred in either of the cultivars 2 days after endophyte inoculation. After 33 days, *lectin* was significantly down-regulated in both cultivars ($t = 6.38$, $P < 0.0001$ and $t = 3.13$, $P = 0.0045$ for cv. Nabusa and cv. Kayinja, respectively) and *PAL* activity significantly up-regulated in the tolerant cv. Kayinja at the same time ($t = 3.88$, $P = 0.0007$). *PAL* activity did not change for the susceptible cv. Nabusa. Similarly, activities of *PR-3*, *PAE*, *PIR7A*, *catalase* and *PR-1* did not change significantly for any of the cultivars.

Apart from *POX*, all the genes tested in this study showed no significant differences in expression levels between the tolerant and susceptible banana cultivars 2 days after endophyte inoculation. *POX* was highly expressed in the tolerant cv. Kayinja compared with its expression in the susceptible cv. Nabusa ($t = 5.83$, $P < 0.0001$). After 33 days, the activities of *PAL* ($t = 5.02$, $P < 0.0001$), *PIR7A* ($t = 3.37$, $P = 0.0043$), *PR-1* ($t = 3.36$, $P = 0.0026$) and *PAE* ($t = 2.19$, $P = 0.039$) were significantly higher in the tolerant cv. Kayinja, while that of *POX* was significantly higher in the susceptible cv. Nabusa ($t = 3.52$, $P = 0.0018$). The expression levels of *lectin*, *PR-3* and *catalase* were not different between the two cultivars 33 days after endophyte inoculation.

The effect of endophyte inoculation or nematode challenge on gene expression differed for both cultivars. In the susceptible cv. Nabusa, endophyte inoculation resulted in higher expression of *lectin*, compared with nematode challenge ($t = 5.51$, $P < 0.0001$) (Fig. 1C). For *POX* the reverse was true. A higher expression was observed following nematode challenge than endophyte inoculation ($t = 2.98$, $P = 0.0067$) (Fig. 1A). Endophyte inoculation or nematode challenge had similar effects on the expression of the other genes in the susceptible cultivar. For the tolerant cv. Kayinja, the expression of *POX* was

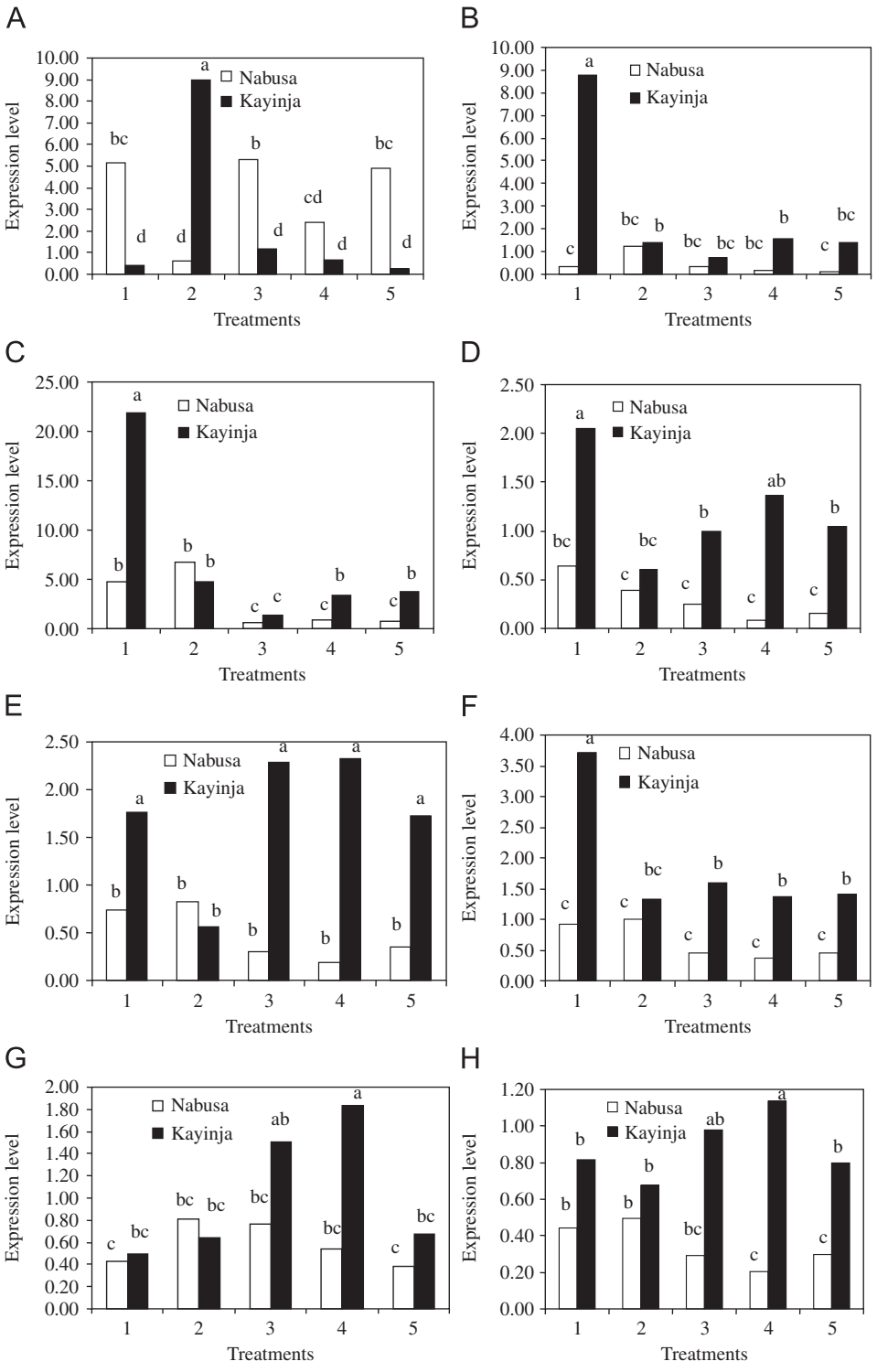

Fig. 1. Expression of defense-related genes in roots of banana cultivars susceptible (Nabusa, genomic group AAA-EA) and tolerant (Kayinja, genomic group ABB) to *Radopholus similis*. Treatment 1—non-inoculated plants (0 h), 2—plants inoculated with non-pathogenic *Fusarium oxysporum* strain V5w2 at 2 days after inoculation (dai), 3—plants inoculated with strain V5w2 at 33 dai, 4—plants inoculated with strain V5w2 and challenged with *R. similis* 30 dai and harvested 3 days after nematode challenge, and 5—endophyte-free plants challenged with *R. similis* on day 30 and harvested 3 days later: (A) *peroxidase* (*POX*), (B) *endochitinase* (*PR-3*), (C) *lectin*, (D) *pectin acetylesterase*, (E) *PAL*, (F) *PIR7A* (*peroxidase*), (G) *catalase* and (H) *PR-1* genes. Bars carrying different letters are significantly different at $P \leqslant 0.05$ (Tukey's studentized range test).

higher 2 days after endophyte inoculation compared with its expression after nematode challenge ($t = 6.68$, $P < 0.0001$). However, nematode challenge in the same cultivar resulted in higher expression of *PAL*, compared with endophyte inoculation ($t = 2.60$, $P = 0.016$) (Fig. 1E). Endophyte inoculation or nematode challenge had similar

effects on the expression of other genes in the tolerant cultivar.

The tolerant cv. Kayinja inoculated with endophytic non-pathogenic *F. oxysporum* and challenged with *R. similis* resulted in the up-regulation of some genes. Although insignificant, the activities of *PR-3* (Fig. 1B), *PAE* (Fig. 1D), *catalase* (Fig. 1G) and *PR-1* (Fig. 1H) were increased by 53, 26, 17 and 14%, respectively, when compared with endophyte-inoculated and nematode non-challenged plants. In contrast, the expression level of *POX* was reduced significantly by 55% ($t = 2.23$, $P = 0.035$) in the susceptible cv. Nabusa. Gene expression between the tolerant and susceptible cultivars was also different after nematode challenge of endophyte-inoculated plants. The expression of *lectin* ($t = 2.36$, $P = 0.027$), *PAE* ($t = 3.35$, $P = 0.0028$), *PAL* ($t = 4.80$, $P < 0.0001$), *PIR7A* ($t = 2.43$, $P = 0.024$), *catalase* ($t = 2.80$, $P = 0.010$) and *PR-1* ($t = 4.07$, $P = 0.0004$) was significantly higher in the tolerant cv. Kayinja, compared with the susceptible cv. Nabusa, while expression levels of *PR-3* increased insignificantly. Expression of *POX* was non-significantly lower in cv. Kayninja than in the susceptible cv. Nabusa. When endophyte-inoculated cv. Kayinja plants were compared to non-endophyte-inoculated plants challenged with *R. similis* after 30 days, the activities of *catalase* ($t = 2.81$, $P = 0.010$), *PR-1* ($t = 2.27$, $P = 0.032$), *PAE* and *PAL* were 172, 42, 30 and 26% higher, respectively.

Non-pathogenic *F. oxysporum* endophytes and *R. similis* were recovered readily from the infected banana roots at harvest, validating results obtained in the inoculation experiment. The first strand cDNA synthesized from banana root RNA was of high quality, and its amplification with actin gene primers indicated no genomic DNA contamination (results not shown).

4. Discussion

The constitutive expression of *PR-3*, *lectin*, *PAL*, *PAE* and *PIR7A* was higher in the tolerant cv. Kayinja, compared with the susceptible cv. Nabusa. Interestingly, *POX* expression constitutively was higher in the susceptible cultivar. Unlike the above genes, there was no significant difference in the constitutive expression of *catalase* and *PR-1* between the tolerant and susceptible cultivars. The differences in constitutive gene expression might explain why cv. Kayinja is more tolerant to *R. similis* than cv. Nabusa, as *PAL* and *PAE* both contribute directly or indirectly to cell wall strengthening [58,59], and the PR-proteins are known to be associated with plant defense against biotic stresses such as pathogen attack [60].

Constitutive expression of *PR-3*, *lectin*, *PAE*, *PAL* and *PIR7A* were significantly down-regulated in the tolerant cv. Kayinja, and that of *POX* in the susceptible cv. Nabusa, 2 days after root infection by the endophytic non-pathogenic *F. oxysporum* strain. The reasons for these down-regulations are unclear, but one can presume that the down-regulated genes are not required for the mutualistic relationship between the endophyte and banana, or their strong down-regulation is necessary for the establishment and development of an endophyte-banana symbiosis. *PAL* and *POX* are enzymes involved in the phenylpropanoid pathway that leads to the synthesis of defense-related phenolics such as lignin and phytoalexins. An increase in their activity is associated with wounding and abiotic and biotic stresses [58]. *Lectins* are unspecific defense proteins produced to act against herbivorous higher animals and phytophagous invertebrates such as plant nematodes with the ability to bind to foreign glycans [61]. Suppression of defense genes has previously been reported in similar symbiotic plant-fungi interactions such as mycorrhiza. For example, Kapulnik et al. [62] reported suppression of phenypropanoid pathway enzymes during establishment of the mycorrhizal fungus *Glomus intraradices* (Schenck and Smith) in alfalfa (*Medicago sativa* L.) and tobacco (*Nicotiana tabacum* L.) roots. In a related study, inoculation of common beans (*Phaseolus vulgaris* L.) with *G. intraradices* reduced *PR-3* activity in roots of mycorrhizal plants compared with non-mycorrhizal plants [63].

Transient expression of *POX* was observed following colonization of the tolerant cv. Kayinja by non-pathogenic *F. oxysporum* in our study, but the increased level had decreased significantly by 33 days after inoculation. Transient expression of *POX* and *catalase* activity was previously reported for the interaction between the mycorrhizal fungus *G. mossea* (Nicol. and Gerd.) and *N. tabacum* [64]. In tobacco, expression of these two genes was up-regulated during appresoria formation, but the increased expressions were later reduced to levels similar to that in non-inoculated plants, similar to our findings. This indicates that the initial plant reaction towards colonization by fungal endophytes is a defense response, as peroxidase is known as a key enzyme in the early oxidative response of plants to pathogens [64]. Whether this response is sustained or not depends on the subsequent activity of the fungus within the plant. It should, however, be noted that a reduced expression of the *POX* gene may occur while the activity of peroxidase remains high in the plant.

The significant up-regulation of *PAL* and non-significant up-regulation of *catalase*, *PR1* and *PAE* in the nematode-tolerant banana cv. Kayinja 33 days after endophyte inoculation indicates that these genes might be involved in the protection of banana plants against pathogen attack. An increased *catalase* activity after endophyte infection points to its involvement in signal transduction during plant-defense [65]. *Catalase* is a tetrameric iron porphyrin necessary for plants to control fluctuating levels of reactive oxygen species under stressful conditions [66]. As a consequence of the role it plays, *catalase* is a well-known signal molecule leading to SAR in plants [67]. Lignin is formed in the phenylpropanoid pathway, and the first step in this pathway is the deamination of phenylalanine to cinamic acid and is catalyzed by the enzyme phenylalanine ammonia-lyase (PAL). Other than lignin, PAL is a precursor for phenylpropanoid-derived secondary plant

products such as salicylic acid and isoflavonoid phytoalexins that are involved in defence [24]. Pectin cell walls have high contents of C_2 and C_3 acetyl esters, which impart physical, chemical and functional properties. PAE hydrolyzes acetyl esters in the homogalacturonan regions of pectin, thereby modifying cell walls especially during root development and pathogen attack [59]. Increased *PAE* activity has been reported in *Arabidopsis thailana* (L.), following infection by root-knot and cyst nematodes [68]. Increased expression of *catalase* and *PAE* was reported in response to *Fusarium* infection for the incompatible reaction between a fusarium wilt-tolerant Cavendish banana selection (GCTCV-218) and *Foc* [53]. Similarly, increased *catalase* activity was reported for the interaction between chickpeas (*Cicer arietinum* L.) and *F. oxysporum* f. sp. *ciceris* [69]. The early expression of *PR-1* in tolerant crop cultivars following pathogen attack is well documented. For example, expression of *PR-1* was induced in pear (*Pyrus pyrifolia* (Nakai) and *P. communis* L.) against infection by Japanese pear scab (*Venturia nashicola* Tanaka and Yamamoto) [60], and in the tolerant banana cv. GCTCV-218 upon *Foc* challenge [53]. *PR-1* proteins, as with other PR proteins, are well known markers of SAR. Although their specific function is not clear, *PR-1* has been associated with antifungal properties such as the hydrolysis of fungal cell walls [32].

For three genes, *R. similis* challenge of endophyte-inoculated plants resulted in a further up-regulation of expression levels observed 33 DAI. A non-significant up-regulation was observed for *PAE*, *catalase* and *PR-1* following *R. similis* challenge of endophyte-inoculated plants of the nematode-tolerant cv. Kayinja, compared with endophyte-inoculated only plants of the same cv. at 33 DAI. For *catalase* and *PR-1*, this expression was significantly higher than that for non-inoculated plants challenged with *R. similis*. This means that the endophyte-induced state in the plant enabled it to respond more effectively to *R. similis* challenge. This phenomenon has been reported for other inducers of plant defense such as SA and the biocontrol fungus *Trichoderma asperellum* strain T203. Treatment of parsley (*Petroselinum crispum* L.) cells with SA elicited COA ligase (4CL) a major gene encoding key enzymes in the phenylpropanoid pathway. A further up-regulation in gene activity was observed following infection with the pathogen *Phytophthora megasperma* f. sp. *glycinea* [70]. Similarly, β-1,3-glucanase activity was up-regulated in cucumber (*Cucumis sativus* L.) 48 h after inoculation with *T. asperellum*. Challenge of *T. asperellum* inoculated plants with *Pseudomonas syringae* pv. *lachrymans* resulted in a significant up-regulation of β-1,3-glucanase activity compared with *T. asperellum* inoculated only and non-inoculated-pathogen infested plants [71]. The observation made for *PAE*, where gene activity down-regulated following colonization by non-pathogenic *F. oxysporum* was up-regulated after pest challenge has been reported. The activity of *PR-10* in tall fescue (*Lolium arundinaceum* Schreb.) was down-regulated following infection with the fungal endophyte *Neotyphodium coenophialum* (Morgan-Jones and Gams), but challenge with the grey leaf spot fungus *Pyricularia grisea* (Cooke) Sacc. resulted in its up-regulation [72].

The up-regulation of defense-related genes in plants inoculated with non-pathogenic *F. oxysporum* and challenged with *R. similis* was observed only in the tolerant banana cv. Kayinja, and not in cv. Nabusa. *F. oxysporum* strain V5w2, however, has been reported to suppress *R. similis* in the susceptible banana cv. Nabusa in screen-house pot trials [45]. It is therefore possible that other genes are induced by strain V5w2 in the susceptible cultivar, which still requires identification. Another possibility is that up-regulation of the genes screened in the current study occurs much later than 3 days post nematode challenge, as was reported in okra (*Abelmoschus esculentus* L. Moench.) where *PAL* activity increased only 15 days after nematode challenge following spraying with salicylic acid [73].

Our study provides the first report of endophyte-induced defense-related gene expression in banana. Though there is indirect evidence that non-pathogenic *F. oxysporum* endophytes can act against *R. similis* through induced resistance, little is known about the resistance pathway and molecules involved. We report endophyte potentiation of the activities of two well known defense-related genes (*catalase* and *PR-1*), for greater expression upon *R. similis* challenge in the tolerant cv. Kayinja. Of much interest also was the significant early down-regulation of certain defense-related genes (*POX*, *PR-3*, *PAE*, *PAL* and *lectin*) upon inoculation with a non-pathogenic *F. oxysporum* endophyte.

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