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Azadirachta indica Reduces Black Sigatoka in East African Highland Banana by Direct Antimicrobial Effects against *Mycosphaerella fijiensis* without Inducing Resistance

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Abstract

Black Sigatoka is a major disease of East African highland cooking bananas in Uganda. Aqueous extracts of *Azadirachta indica*, *Cinnamomum zeylanicum* and *Capsicum annuum* have shown the potential to reduce Black Sigatoka in banana plantlets. The mechanisms by which plant extracts confer protection against plant pathogens has previously been reported to involve activation of defence and direct antimicrobial activity. In the current study, both antimicrobial activities of selected extracts were studied as well as expression of three defence-related genes using quantitative real-time PCR. Gene expression was compared in susceptible (cv. Musakala, genomic group AAA-EA) and resistant (cv. Kayinja, genomic group ABB) banana cultivars. Additionally, Musakala treated with *A indica* extract at 1 day before inoculation (DBI) was tested for induction of defence-related genes at 0, 10 and 20 days after inoculation (DAI). *Pathogenesis-related genes* (*PR-1* and *PR-3*) and *non-expressor of PR-genes* (*NPR1B*) were up-regulated in the resistant cultivar. The genes analysed responded at late time points to *M. fijiensis* inoculation in both extract-treated and control plants in the susceptible cv. Musakala. On the other hand, *A. indica* and *C. annuum* completely inhibited mycelial growth of *M. fijiensis* at 30% (w/v). These findings suggest that the effect of plant extracts on Black Sigatoka is strongly associated with the direct antimicrobial effects.

Keywords: banana, plant extracts, Black Sigatoka, defence genes

1. Introduction

Bananas and plantains constitute major tropical food crops and Black Sigatoka, caused by the fungus *Mycosphaerella fijiensis*, is economically the most destructive foliar disease of bananas and plantains worldwide (Friesen, 2016; Portal et al., 2011). *M. fijiensis* is a highly diverse hemibiotrophic fungus (Churchill, 2011), which induces foliar leaf streaks that considerably reduce the photosynthetically active leaf area. Estimates of losses to Black Sigatoka for dessert bananas and plantains are in the range of 20-80% in the absence of fungicides (Churchill, 2011; Anonymous, 2009).

In its biotrophic phase, *M. fijiensis* exclusively colonises the intercellular spaces between mesophyll cells and obtains nutrients from the host apoplast without formation of haustoria. The fungus stays in this phase for 3-4 weeks before any necrotic lesions appear (Lepoivre et al., 2003). When the apoplast is depleted of nutrients, the fungus switches to the necrotrophic phase in which it is predicted that toxins targeting the chloroplasts play an important role (Harelimana et al., 1997). *M. fijiensis* starts producing conidia in the early necrotrophic phase. The production of sexual spores, however, does not start until the final infection stage, in completely necrotic lesions (Bennett & Arneson, 2003). Knowledge derived from plant-pathogen interaction studies is highly important for the rational selection of genotypes resistant to plant pathogens (Lepoivre et al., 2003). Important aspects of the interactions between *Musa* spp. and *M. fijiensis* are still unknown (Timm et al., 2016; Portal et al., 2011). The

current information in the literature regarding both fungal (Cho et al., 2008) and plant genes (Khayat, 2004) that are involved in the interaction between *M. fijiensis* and *Musa* spp. is poor. Therefore, detailed molecular studies of this host-pathogen interaction are necessary for a better understanding of the events that take place during the infection processes (Lepoivre et al., 2003).

Investigations on mechanisms of disease suppression by plant products have suggested that the active principles present in them may either act on the pathogen directly (Hubert, 2015; Amadioha, 2000) or induce resistance in the host plant, resulting in reduction of disease development (Baysal & Zeller, 2004). However, the effect depends on the type of extract. Guleria and Kumar (2006) reported that an aqueous leaf extract of neem (*Azadirachta indica*) provided control of the Alternaria leaf spot pathogen (*Alternaria sesame*) of sesame (*Sesamum indicum*) through induction of the enzymes phenylalanine ammonia lyase (PAL), peroxidase (POX) and phenolic compounds. In a related experiment done by Bengtsson et al. (2009), comparing the effects of a yucca extract and acibenzolar-S-methyl (ASM) on inhibition of *Venturia inaequalis* in apple leaves, observations suggested that the yucca extract acted mainly by a direct fungitoxic effect whereas ASM acted as a resistance inducer. Expression studies of two genes encoding the PR-proteins, *PR-1* and *PR-8*, in apple seedlings indicated that yucca extract also activated plant defence since expression of both genes was up-regulated following yucca treatment, to a level similar to that observed after treatment with ASM. Additionally, Pal et al. (2011) reported that seed treatment of rice seeds with leaf extracts of *Cymbopogan citrus* and *Ocimum sanctum* increased accumulation of defence-related enzymes (PAL, catalase, POX, polyphenol oxidase and tyrosinase) in plants raised from the treated seeds compared to control seeds. Kagale et al. (2011) demonstrated that foliar application of aqueous leaf extracts of *Zizyphus jujuba* and *Ipomoea carnea* followed by challenge inoculation with *Rhizoctonia solani* induced resistance in rice. This was confirmed from the increased activities of the enzymes chitinase, β-1,3-glucanase and POX, as well as increased accumulation of defence-related compounds such as PAL and phenolic substances. This makes it clear that plant extracts can induce resistance in plants against pathogens as well as providing a direct antimicrobial effect.

The current study was designed to elucidate the mode of action of the aqueous extracts from *Azadirachta indica, Cinnamomum zeylanicum* and *Capsicum annuum* against *M. fijiensis* in a susceptible East African Highland banana (EAHB) cultivar Musakala (genomic group AAA-EA). *Azadirachta indica*, *Cinnamomum zeylanicum* and *Capsicum annuum* were selected for this study based on the results of prelimanry experiments in which 15 extracts were tested, and others rejected. Specifically, the aim was to test if direct antimicrobial effects and induced resistance were involved.

2. Materials and Methods

2.1 Preparation of Plant Extracts and Fungal Inoculum and Cultivation of Banana

Leaves of *Azadirachta indica*, bark of *Cinnamomum zeylanicum* and fruits of *Capsicum annuum* were washed with running tap water to remove soil contaminations, followed by rinsing with sterile distilled water three times. The samples were placed on benches in a screen house (temperature 22-28 °C) to dry for 7 days. The dried sample of each plant species was ground thoroughly into powder using a blender (Hamilton Beach Brands, Inc., USA). *Mycosphaerella fijiensis* (local isolate Mak01) was grown on potato dextrose agar (Sigma-Aldrich, USA) at 25 °C. Agar plugs (3 mm in diameter) from actively growing 14-day-old cultures were aseptically transferred to malt extract agar plates (Sigma-Aldrich, USA). The inoculated plates were sealed and incubated at 25 °C in an incubator for 21 days in darkness. Mycelium from each plate was scraped off with a sterile scalpel, bulked and weighed on pre-weighed sterile filter paper. The weighed mycelium was fragmented in a blender (Amazon, USA) at full speed for 3 min. A mycelium master suspension was prepared and diluted to a final concentration of 15 mg/ml.

Two month old tissue culture banana plantlets of cv. Musakala from Makerere University Agricultural Research Institute tissue culture laboratory were transplanted to individual pots containing pre-sterilized loam soil. Banana plantlets were grown in the screen house at a temperature of 22-28 °C under natural light conditions for one month. Plantlets were used for experiments when they had 4-6 fully expanded leaves.

2.2 Tests for Direct Antimicrobial Activity of Plant Extracts

An aqueous extract concentration of 20% (w/v) was used to test for fungicidal activity under laboratory conditions. Three treatments (immersion of a 3-mm fungal plug obtained from an actively growing fungal culture on PDA separately for 1, 2 or 4 h in each extract) were randomly applied to 12 fungal plugs for each extract. A fungicide, difenoconazole at 0.1% (v/v, Syngenta, Kenya) was included for comparison. Fungal plugs were washed with sterile distilled water three times prior to inoculation on PDA plates to remove the crude extracts. Fungal blocks which were dipped in sterile distilled water for 1, 2 or 4 h were included as untreated

controls. Treated fungal blocks were placed aseptically in the centre of 9 cm diameter Petri dishes, sealed and incubated at room temperature (25 °C) for 28 days. Colony growth was determined by measuring the diameter using a mathematical ruler. Percentage inhibition of mycelial growth was calculated using the formula: percentage inhibition = $100 \times (1 - ab^{-1})$, with a = colony diameter for the treatment and b = colony diameter for the untreated control. A completely randomized design with 4 replications was adopted for the trial. The experiment was carried out twice.

Additionally, qualitative phytochemical examinations of the extracts were carried according to the procedure described by Prashanth et al. (2011) to profile the antimicrobial compounds present in the extract. To detect the presence of phenols, 1.0 ml of 10% (w/v) aqueous leaf extract of *A. indica*, fruit extract of *C. annuum* and bark extract of *C. zeylanicum* were treated with 3-4 drops of 1% (w/v) ferric chloride solution. Formation of bluish, black colour indicated the presence of phenols. For flavonoids, 1.0 ml 10% (w/v) extracts were treated with 3 drops of sodium hydroxide (1.2 M) solution in a test tube and formation of intense, yellow colour, which became colourless on addition of 0.1 ml of 0.1 M hydrochloric acid, indicated the presence of flavonoids. Wagner's reagent (2 g iodine and 6 g potassium iodide in 100 ml water) was used to test for alkaloids. To 1.0 ml of 10% (w/v) extract, 3 drops of Wagner`s reagent were added on the side of the test tube. Formation of brown/reddish precipitate indicated the presence of alkaloids. For tannins, 2-3 drops of ferric chloride solution (0.1 M) was added to 1.0 ml 10% (w/v) extract in a test tube. A dark green colouration indicated the presence of tannins. For saponins, 1 ml 10% (w/v) aqueous extract was added to 0.1 ml of distilled water in a test tube and shaken vigorously. A stable persistent froth for 20 min confirmed the presence of saponins. The experiment was performed twice with different batches of extract.

2.3 In Planta Tests for Effects of Plant Extracts under Controlled Conditions

Screen-house pot experiments were set up with tissue cultured banana plantlets using cooking banana cultivars Musakala, Mbwazirume, Mpologoma and Kibuzi as susceptible genotypes. In the first trial, three treatments (0, 10, 30% (w/v) of *A. indica* extract from the Lira district (Northern Uganda), the Mbale district (Eastern Uganda) and the Wakiso district in the central region of Uganda) were applied two days before inoculation (DBI) with *M. fijiensis* to test the effect of extracts made from plants harvested from different growth conditions. Treatments were randomly applied to 12 plants of Musakala for each extract growth condition. Disease was assessed at 45 DAI. To assess disease, number of lesions (severity) was counted.

In the second trial, banana cultivars Mpologoma, Mbwazirume and Kibuzi (susceptible to *M. fijiensis* in Uganda) were used to compare effect of the extracts on different banana cultivars. After preliminary tests using 10, 20 and 30% extract concentrations, 30% (w/v) extract was chosen for further studies since it was more efficacious than the 20% concentration used in the in vitro tests. Different spraying time points (0, 2, 4 or 6 DBI) were tested to select the optimal time of application. Disease was assessed at 45 DAI to determine how long the extract could reduce symptoms. To test whether the disease-reducing effect was dependent on induced resistance, an experiment was also performed in which treated and non-treated leaves on the same plant were inoculated. Four spraying time points (0, 2, 4 and 6 DBI) were tested and the disease was assessed at 45 DAI. For all experiments, a completely randomized design was adopted. Each experiment was performed at least twice.

2.4 Test for Effect of A. indica on Sporulation of M. fijiensis

To quantify the effect of *A. indica* on ascospore production of *M. fijiensis*, 5 cm long leaf segments were cut from banana leaves treated with *A. indica* (30% [w/v]) extract 0, 2, 4 and 6 DBI 45 days after inoculation. Percentage infected leaf area for each treatment was recorded. The experiment was conducted as fully randomized block experiment with three replications. Nine samples were obtained for each treatment. The leaves were observed under a stereo microscope for presence of pseudothecia. Pieces of the leaf containing three lesions with mature pseudothecia were cut and incubated in polythene bags with moist cotton wool for 48 h. The incubated samples were surface sterilised with 0.5% sodium hypochlorite for 5 min followed by 70% ethanol for 5 min. The treated leaf samples were serially rinsed in sterile distilled water, and then stapled onto sterile filter papers. The filter papers were ascetically transferred to lids of Petri dishes and left to discharge ascospores over potato dextrose broth (10 ml) for 6 h. For each sample, ascospore concentration was determined in two subsamples using a haemocytometer.

2.5 Tests for Gene Expression to Verify Induction of Resistance

Screen-house pot experiments were set up with tissue culture banana plantlets using cv. Psang awak and the cooking banana cv. Musakala as resistant and susceptible genotypes, respectively. Tissue culture plants of each banana genotype were transplanted in plastic pots and allowed to establish for 8 weeks. The two-month-old seedlings were transplanted to individual pots containing pre-sterilized loam soil (pH 6.9). Plantlets were grown

in the screen-house at a temperature of 22-28 °C under natural light conditions for one month and were used for experiments when they had 4-6 fully expanded leaves. A completely randomized design was adopted for the trials.

In the first trial, two treatments (inoculation with either *M. fijiensis* or spraying with water) were randomly applied to 24 plants of each genotype to study the expression of the target genes in a tolerant and susceptible banana without extract treatment. *M. fijiensis* inoculum of approximately 15 mg mycelium/ml was prepared using isolate Mak01, preserved at the Makerere University Agricultural Research Institute, Kabanyolo, Uganda. A volume of 0.5 ml of inoculum (5 mg/ml) or water was applied on the abaxial surfaces of the first and second fully unfolded leaves until run-off, using a painter's brush. A random leaf sample (10 g) of three plants for each treatment was harvested at 0, 1, 5, 9, 13, 19, 21 and 25 DPI for RNA extraction. In the second trial, four treatments (spraying with extract of *A. indica*, water, inoculation with *M. fijiensis* and spraying with extract of *A. indica* plus inoculation with *M. fijiensis*) were applied to nine plants for each treatment. Inoculation was performed as for the first trial. Random leaf samples (10 g) of three plants for each treatment was harvested at 0, 10 and 20 DPI for RNA extraction.

2.6 RNA Extraction

RNA from each sample was extracted following the CTAB nucleic acid extraction protocol (Ssekiwoko et al., 2015). All materials (centrifuge tubes, pipette tips) used were RNase free. The mortars and pestles were sterilized by soaking in 2% H_2O_2 overnight, then in 1 M NaOH for 1 h, rinsed in distilled water, baked at 140 °C for 2 h, cooled and wiped with DEPC treated water. Generally, 0.1 g of each frozen plant tissue was crushed into a powder in a sterile and frozen mortar and 1.5 ml pre-warmed (65 °C) extraction buffer was added to a test tube, which was incubated at 65 °C for 15 min followed by centrifugation at 7,558 g for 5 min. A volume of 700μl supernatant was transferred to a new sterile tube and 700 μl chloroform: isoamyl alcohol mixture (24:1, v/v) was added and mixed by inversion and then centrifuged at 7,558 g for 5 min at 4 °C. The aqueous phase (600 μl) was transferred to a new tube and an equal volume of a mixture of phenol: chloroform: isoamyl alcohol (25:24:1, v/v) added, mixed by inversion and then centrifuged at 7558 g for 5 min at 4 °C. Finally, 500 μl of the aqueous phase was transferred to a new tube and RNA precipitated by adding 10 M lithium chloride to a final molarity of 2.25 M followed by incubation at -20 °C for 8 h. The precipitate was then collected by spinning at 7,558 for 45 min. The pellet was dissolved in water to 450 μl, then 50 μl 3 M sodium acetate (pH 5.2) was added, followed by an equal volume of cold isopropanol (-20 °C) and the tube mixed by inversion. The mixture was kept at -20 °C overnight and then spun at 7,558 g for 2 min at 4 °C. The pellet was washed twice in 500 μl 70% (v/v) ethanol by tapping and spinning at 7,558 g for 2 min at 4 °C, air dried at room temperature for 40 min followed by re-suspension in 30 μl sterile nuclease free water (Fermentas, USA). The concentration was determined using a NanoDrop 2000 spectrophotometer (Wilmington, DE, USA) and the RNA stored at -20 °C. Genomic DNA traces were removed from the extracted RNA using a DNase treatment kit (Thermo Scientific, USA) following the manufacturer's protocol and then stored at -20 °C awaiting cDNA synthesis.

2.7 cDNA Synthesis

cDNA was synthesized using the RevertAid™ first strand cDNA synthesis Kit (Fermentas Life Sciences), according to manufacturer's direction, using 2.5 ng/ml random hexamers for priming. To 8 μl nuclease free water, 3 μl pure RNA sample and 1 μl random hexamer primers (0.2 μg/μl) were added. This mixture was incubated at 65 °C for 5 min, then immediately chilled on ice for 5 min and the content collected by a brief spinning. During reverse transcription, 4 μl 5 × reaction buffer, 1 μl Ribolock RNase inhibitor (20 U/μl), 2 μl 10 mMdNTP mix, and 1 μl reverse transcriptase (200 U/μl) was added to the primer annealing reaction mixture. This mixture was incubated at 25 °C for 5 min followed by incubation at 42 °C for 1 h and heating at 70 °C for 5 min to inactivate the reverse transcriptase. The cDNA was then chilled on ice, collected by spinning and quantified using a NanoDrop 2000 spectrophotometer, followed by dilution to 0.1 μg/μl using sterile nuclease free water before storage at -20 °C. The quality of the cDNA was verified by amplifying a 200 bp actin fragment with banana actin (house keeping gene) forward 5'-ACCGAAGCCCCTCTTAACCC-3' and reverse 5'-GTATGGCTGACACCAT CACC-3' primers.

2.8 Real-Time RT-PCR Primers

The expression profile of three banana defence-related genes was analysed in cDNA samples obtained from banana leaves. Primers for the genes are seen in Table 1. One of these genes, *PR-1*, was previously reported to be up-regulated in a Cavendish selection (genomic group AAA) tolerant to *Fusarium oxysporum* f. sp. *cubense* (*Foc*) (Van den Berg et al., 2007). *PR-3*, was found to be up-regulated following *Foc* challenge of cv. Lady Finger (genomic group AAB) and cv. Cavendish plants previously inoculated with non-pathogenic *F. oxysporum*

(Forsyth, 2006). An endogenous gene, *Musa 25s* (Van den Berg et al., 2007), was used as a house-keeping gene (Endah et al., 2010). Primer sequences for the amplification of *PR-1*, *PR-3* and *Musa 25s* were obtained from Van den Berg et al. (2007), whereas for *non-expressor of pathogenesis-related protein* (*NPR1B*), primer sequences came from Endah et al. (2010). *MNPR1B* expression is associated with increased *PR* gene expression in disease tolerant banana cultivar GCTCV-218 (Endah et al., 2010). All primers were synthesized by Bioneer Corporation (Munpyeongseo-ro, South Korea).

Table 1. Primer sequences of defence-related genes studied in banana cultivars tolerant (Kayinja, genomic group ABB) and susceptible (Musakala, genomic group AAA-EA) to *Mycosphaerella fijiensis* by quantitative real time-PCR following treatment with *Azadirachta indica* and challenge with *M. fijiensis*

Primer	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')	Product size (bp)
Musa 25s[A]	ACATTGTCAGGTGGGGAGTT	CCTTTTGTTCCACACGAGATT	106
PR3[A]	GGCTCTGTGGTTCTGGATGA	CCAACCCTCCATTGATGATG	150
PR1[A]	TCCGGCCTTATTTCACATTC	GCCATCTTCATCATCTGCAA	126
NPR1B[B]	AGGTTTGCCCGAACAAGAAG	TGAGAGGCAACAACTCAGAGAG	150

Note. A from Van den Berg et al. (2007), B From Endah et al. (2010).

2.9 Quantitative RT-PCR

Gene expression analysis was performed in two technical replications for each of three biological replications, using a 7500 Applied Biosystem qPCR system with 150 ng cDNA and 10 ml of AccuPower® 2X Greenstar qPCR Master Mix (Bioneer Corporation, South Korea). Each PCR reaction consisted of 5 µl diluted template (30 ng/µl), 0.2 µM primers, and 10 µl AccuPower® 2X Greenstar qPCR Master Mix. The reaction volume was adjusted to 20 µl with nuclease-free water. Non-template control (NTC) reactions contained water instead of cDNA as template. Cycling consisted of an initial denaturation phase of 7 min at 95 °C, an amplification phase of 30 cycles each consisting of a denaturation step at 95 °C for 10 s, annealing at 63 °C for 5 s and extension at 72 °C for 30 s and data acquisition at 95 °C. Analysis of melting curves was carried out at the end of each run to evaluate undesired amplifications. Expression of the *Musa 25s rRNA* gene that was previously evaluated to be constitutively and constantly expressed in *Musa* spp. (Endah et al., 2010) was used as to normalise target gene data.

2.10 Data Analysis

The data on the effect of treatments on the growth of pathogens and Black Sigatoka severity scores, were analysed by analysis of variance assuming a normal distribution. Variances were stabilised by appropriate transformation of data if necessary. Data were analysed by PC-SAS (release 9.2, SAS Institute, Cary, NC). For the gene expression studies, temporal expression of each selected gene at all-time points were compared to its expression at 3 h (day 0) using the $2^{-\Delta\Delta CT}$ relative gene expression method (Livak & Schmittgen, 2001). Hypotheses were rejected at $P \leq 0.05$.

3. Results

### 3.1 Direct Antimicrobial Activity of Plant Extracts against M. fijiensis

The fungicidal activities of extracts of *Azadirachta indica* leaves, *Capsicum annuum* fruits and *Cinnamomum zeylanicum* bark were tested to understand their ability to inhibit the vegetative forms of *Mycosphaerella fijiensis*, which was immersed in extract solutions (20% w/v) for different time periods (1, 2 and 4 h) (Table 2). Direct antimicrobial activities of *A. indica*, *C. annuum* and *C. zeylanicum* were seen on *M. fijiensis*. Indeed, by immersing fungal plugs of actively growing *M. fijiensis* in sterile distilled water and 20% filter-sterilized extracts, inhibition by the plant extract was observed. Percentage inhibition increased with increase in the immersion time. The percentage inhibition at 4h of immersion was 87.8, 100 and 52.4% (Experiment 1) and 93.6, 100 and 48.4% (Experiment 2) for *Azadirachta indica, Capsicum annuum* and *Cinnamomum zeylanicum* respectively. Percentage inhibition increased with increasing immersion times. At 4 h immersion, the percentage inhibition was 87.8, 100.0 and 52.4% (Experiment 1) and 93.6, 100.0 and 48.4% (Experiment 2) for *Azadirachta indica, Capsicum annuum* and *Cinnamomum zeylanicum*, respectively.

Table 2. Fungicidal activity of aqueous extracts of *Azadirachta indica*, *Capsicum annuum* and *Cinnamomum zeylanicum* against *Mycosphaerella fijiensis*

Extract	Percentage inhibition of fungal growth					
	Experiment 1			Experiment 2		
	Immersion time (h)			Immersion time (h)		
	1	2	4	1	2	4
C. zeylanicum	1.2	17.9	52.4	1.5	18.6	48.4
A. indica	53.8	78.3	87.8	69.7	71.4	93.6
C. annuum	67.5	88.7	100.0	71.7	85.7	100.0
Difenoconazole	100.0	100.0	100.0	100.0	100.0	100.0
LSD	21.3	24.6	9.9	25.4	19.1	8.3
P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

3.2 Effect of Foliar Spraying with Azadirachta indica Leaf Extract on Black Sigatoka

Leaf extracts of *Azadirachta indica* grown at different locations in Uganda was used to verify that the protective effect was not dependent on the geographical origin of plant material (Figure 1A). Concentrations of 0, 10 and 30% of the extracts were tested by spraying at 2 DBI. Mean lesion numbers were reduced compared with the control treatment using water at 45 DAI for 30% extract made from plants grown in the North (35.1%), East (30.1%) and 31.9% Central Uganda. Extracts from the three locations did not differ in ability to confer protection at 30% (w/v) extract concentration ($P \leq 0.110$). Within each location, there was a significant effect of extract concentration ($P \leq 0.010$, $P \leq 0.022$ and $P \leq 0.010$ for North, East and Central Uganda, respectively). The effect was subsequently verified in the three EABH cultivars Mpologoma, Mbwazirume and Kibuzi (Figure 1C), using 0, 10 and 30% (w/v) extract from plants grown in northern Uganda. For 30 % extract, significant reductions of mean lesion numbers compared with the control treatment were observed in all the cultivars (35.9% for cv. Mpologoma, 37% for cv. Mbwazirume and 42.2% for cv. Kibuzi) ($P \leq 0.010$).To find the optimal spraying time point and to study how long the extract could reduce symptoms of the disease, four spraying time points (0, 2, 4, and 6 DBI) were tested and the disease was assessed at 45 DAI (Figure 1D). Based on previous results, 30% leaf extract was chosen for this experiment. Spraying time points of 0, 2 and 4 DBI significantly reduced disease (65.8, 41.4 and, 19.0%, respectively) whereas there was no effect for 6 DBI (2.6%).
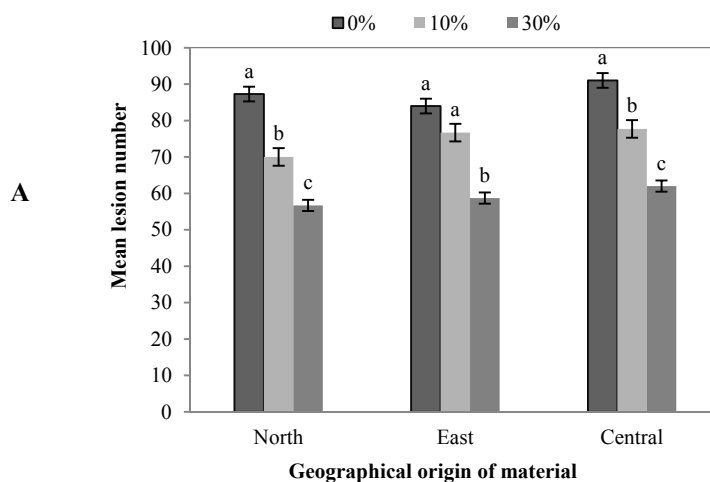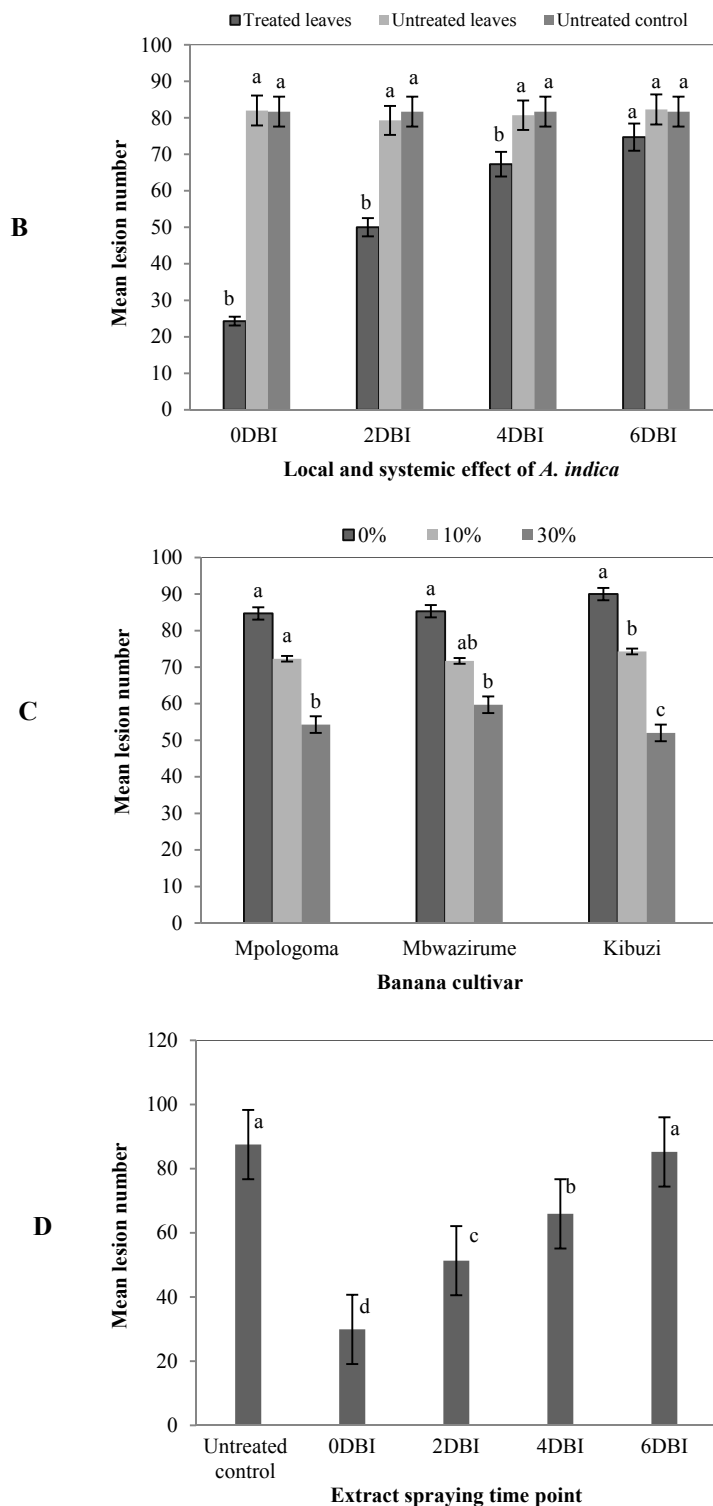
Figure 1. Black Sigatoka development in banana leaves of cvs. Musakala, Mpologoma, Mbwazirume and Kibuzi after treatment with extract of *Azadirachta indica* and challenge inoculation with *Mycosphaerella fijiensis*

Note. (a) Effect of geographical origin of *A. indica* on *M. fijiensis* in banana cv. Musakala; (b) Local and systemic effects of *A. indica* against *M. fijiensis* in cv. Musakala; (c) effect of *A. indica* extract (0, 10 and 30% w/v) on banana cvs. Mpologoma, Mbwazirume and Kibuzi, against *M. fijiensis* when sprayed at 2 DBI; (d) Effect of 30% w/v *A. indica* on ability to control *M. fijiensis* in banana (cv. Mbwazirume) when applied at 0, 2, 4 and 6 DBI. Disease was assessed at 45 days after inoculation. Means followed by the same letter within a column are not significantly different ($P \leq 0.05$).

3.3 Local and Systemic Effects of Azadirachta indica on Infection by M. fijiensis

To test whether the disease-reducing effect was systemic, an experiment was also performed in which extract treated and non-extract treated leaves on the same plant were inoculated with *M. fijiensis*. Four spraying time points (0, 2, 4, and 6 DBI) were tested and the disease was assessed at 45 DAI (Figure 1B). Treatment of the youngest fully opened leaves with *A. indica* extract reduced the average infection level of *M. fijiensis* by 70.3, 38.8, 17.6 and 8.6% for pre-extract treatment timing of 0, 2, 4, 6 DBI respectively (Figure1B). In contrast, no systemic disease-reducing effect was observed on the non-extract primed leaves on the same test plants. No differences were seen between non-extract treated leaves on the test plants and control plants.

3.4 Effect of A. indica on Sporulation of M. fijiensis

Application of *A. indica* extract (30% w/v) reduced the sporulation of *M. fijiensis* (Table 3.). The efficacy depended on the timing of the application of *A. indica*. Three *A. indica* treatments (0, 2 and 4 DBI) reduced the average production of *M. fijiensis* ascospores, with the least reduction when *A. indica* was applied 4 DBI with *M. fijiensis*. The treatment of at 6 DBI did not reduce the mean ascospores production.

Table 3. Disease severity caused by *A. indica* and ascospore production of the pathogen on the leaf of cv. Musakala sprayed with a mycelia suspension (15 mg/ml) of *M. fijiensis* at 6, 4, 2 or 0 (simultaneously) days after extract treatment

Treatment	Disease severity (% leaf coverage)	Mean ascospore production in 3 lesions from disease leaf area
Water, 0DBI	80	3312
A. indica, 6DBI	77.5	2779
A. indica, 4DBI	67.0	2115
A. indica, 2DBI	46.1	1263
A. indica, 0DBI	19.3	1136
LSD	4.9	640
P-value	< 0.001	< 0.001

Note. Means followed by the same letter within a column are not significantly different ($P \leq 0.05$).

3.5 Qualitative Phytochemical Analysis of the Aqueous Extracts of Plant Extracts

The results of qualitative phytochemical analysis of *A. indica* leaf extract, *C. annuum* fruit extract and *C. zeylanicum* bark extract are given in Table 4. There were high levels of tannins and flavonoids and moderate levels of phenolic compounds and alkaloids in *A. indica*. In contrast, *C. annuum* and *C. zeylanicum* extracts only showed moderate level of flavonoids and tannins, respectively. *Azadirachta indica* and *C. annuum* were from the Northern part Uganda and *C.zeylanicum* from the Central part Uganda.

Table 4. Phytochemical components of the aqueous leaf extract of *Azadirachta indica*, fruit extract of *Capsicum annuum* and bark extract of *Cinnamomum zeylanicum*

Constituents	Test	Result		
		Azadirachta indica	*Cinnamomum zeylanicum*	*Capsicum annuum*
Phenolic compounds	Ferric chloride test	++	-	+
Tannins	Ferric chloride test	+++	++	+
Flavonoids	Alkaline reagent test	+++	-	++
Saponins	Froth test	+	+++	++
Alkaloids	Mayer's reagent test	++	-	+

Note. + = Trace, ++ = Moderate, +++ = High, - = Absent.

3.6 Expression of Defence-Related Genes (PR-Proteins) in Response to M. fijiensis Infection

The resistant cv. Psang awak was included to profile the expression of the target genes in a resistant cultivar in response to *M. fijiensis* infection. *PR-1* expression in inoculated plants of cv. Psang awak (Figure 2A) increased significantly from 0 to 1 DAI, indicating an early induction. Subsequently, expression gradually declined until 13

DAI, after which time expression increased significantly again. For cv. Musakala, there was no significant changes in expression compared to mock-inoculated plants. For *PR-3* (Figure 2B), there was no marked expression in inoculated cv. Psang awak plants compared to the control until 13 DAI after which time, expression gradually increased. In the susceptible cv. Musakala there was no change in expression of *PR-3* at any time point studied. The expression of *NPR1* (Figure 2C) followed the same pattern of expression as PR1 in both cvs. Psang awak and Musakala.
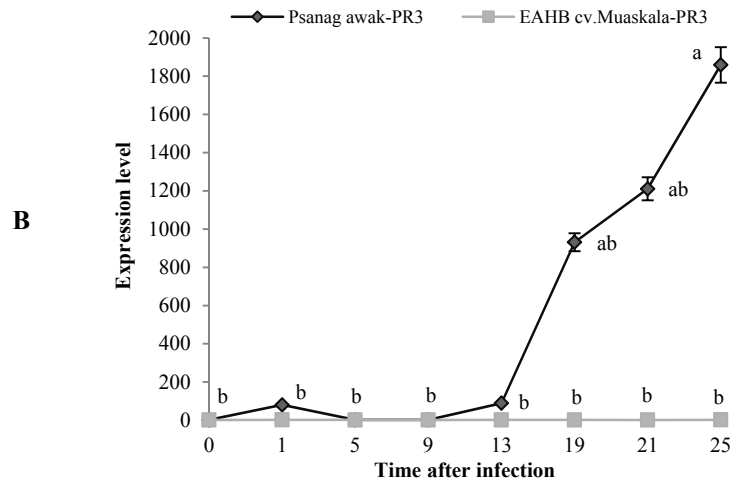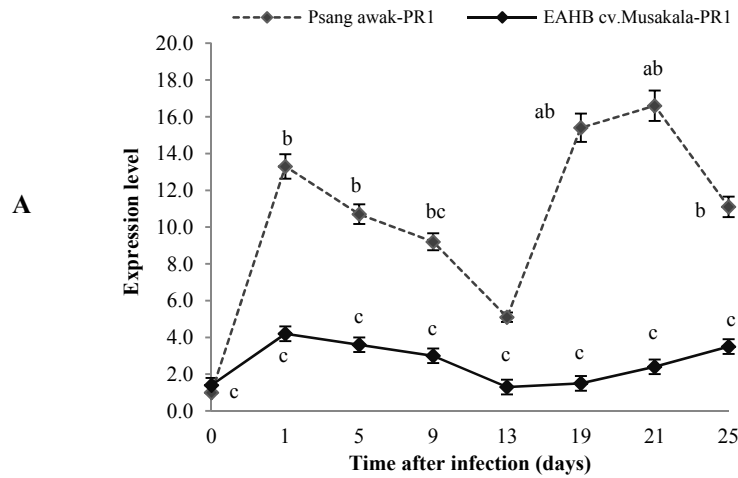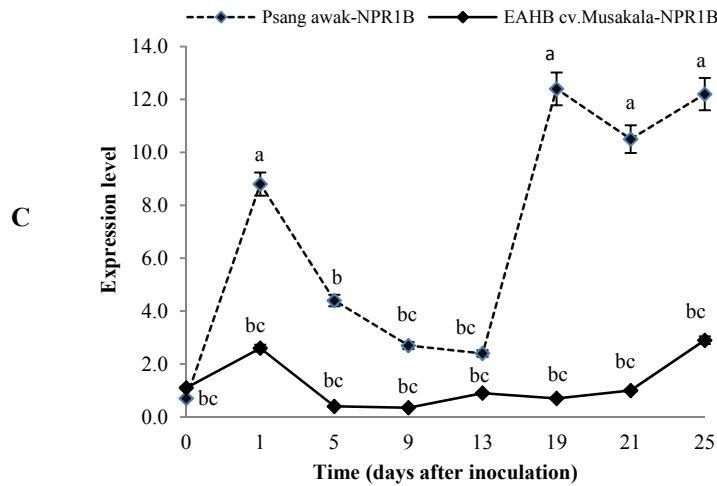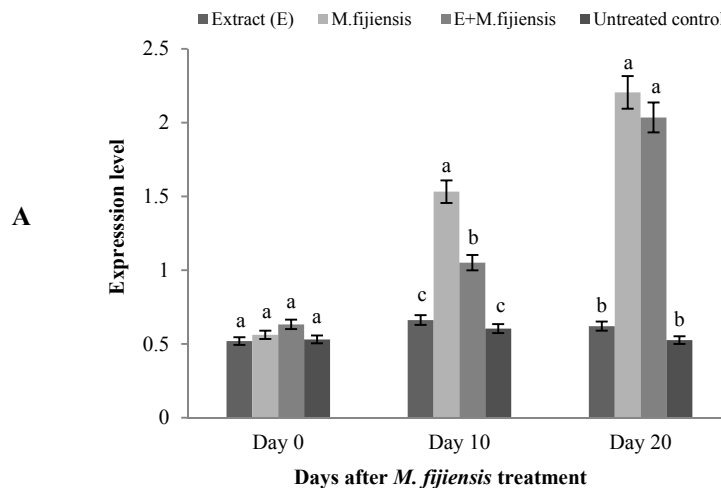
Figure 2. Expression levels of A) *PR-1*; B) *PR-3* and C) *NPR1B* following inoculation of cvs. Psang awak and Musakala with *Mycosphaerella fijiensis* compared to water treated control for each cultivar

Note. Means marked with the same letters are not significantly different at $P \leq 0.05$.

3.7 Expression of Defence-Related Genes of Banana Treated with Azadirachta indica Extract

Expression of the PR-proteins *PR-3* and *PR-1* (Figure 3) was studied in cv. Musakala after treatment with *A. indica* extract. Whereas the extract alone did not result in any alteration of expression of either gene, inoculation with the pathogen, and pre-treatment with the extract followed by pathogen inoculation, resulted in increased expression at 10 and 20 DAI. *PR-1* expression significantly increased in plantlets inoculated with *M. fijiensis* only (2.7-fold) ($P \leq 0.010$) and in plantlets treated with *A. indica* and challenge inoculated with *M. fijiensis* (1.7-fold) ($P \leq 0.001$) 10 days post *M. fijiensis* inoculation (Figure 3A) when compared to the expression at the beginning of the experiment (day 0). Similar observationss were made for day 20 (3.9 fold) for *M. fijiensis* inoculation and 3.2 fold for *A. indica* plus *M. fijiensis*). *PR-3* expression also significantly increased in cv. Musakala treated with *M. fijiensis* alone (1.8 and 3.3-fold ($P \leq 0.010$) for day 10 and 20 respectively) and in plantlets treated with *A. indica* + *M. fijiensis* (1.8-fold and 3.1-fold ($P \leq 0.001$ for day 0 and 20 respectively) when compared to *PR-3* expression at the beginning of the experiment (day 0) (Figure 3B). However, none of the genes showed elevated expression in plants treated with *A. indica* + *M. fijiensis* when compared with plants treated with *M. fijiensis* alone.
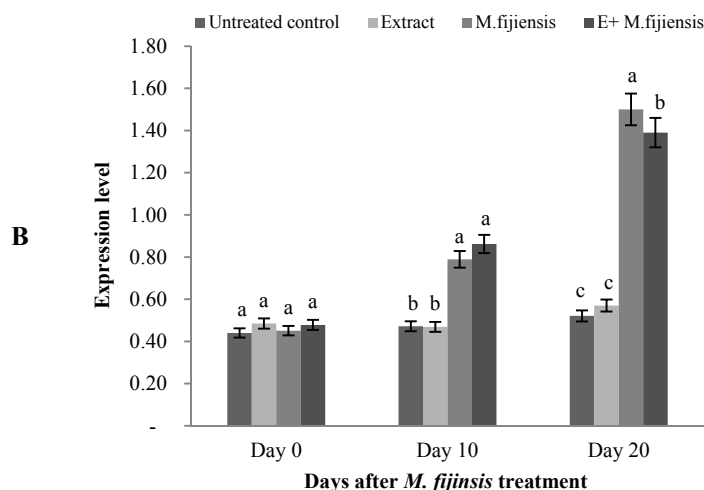
Figure 3. Expression levels of A) *PR-1* and B) *PR-3* in cv. Musakala treated with *A. indica* following inoculation with *M. fijiensis*

Note. Means followed by the same letter within a column are not significantly different ($P \leq 0.05$).

4. Discussion

In the present study, aqueous extract of *Azadirachta indica* was found to possess the ability to protect banana against Black Sigatoka caused by *Mycosphaerella fijiensis* under controlled conditions in Uganda in the three East African Highland banana cultivars, cvs. Mbwazirume, Mpologoma and Kibuzi. Leaf extract of *A. indica* and fruit extract of *Capsicum annuum* completely inhibited the mycelial growth of *M. fijiensis* under *in vitro* conditions at a concentration of 30% (w/v). Extracts of *A. indica* from dry leaves grown in different regions in Uganda were able to significantly reduce disease. Plants of the same species grown under different conditions could vary, for example, in secondary metabolite production and therefore, in their ability to control the disease, but in the present study, the active components were maintained in plants grown under different conditions. Additionally, both fresh (data not presented) and dried leaves of *A. indica* were able to reduce the disease, showing that the active components of the extract still maintained their effect after drying. Although *A. indica* is a widely grown tree in Uganda whose fresh leaves could be easily obtained, using dried leaves potentially represents a useful solution for farmers in areas where the plant is not abundant.

Management of several fungal (*Alternaria solani*, *Fusarium oxysporum*, Hassanein et al., 2008; Sallam, 2011), bacterial (*Xanthomonas campestris*, Reddy, 2012) and nematode diseases (*Meloidogyne javanica*, *Meloidogyne incognita*) (Javed et al., 2008; Hadian et al., 2011) in greenhouse and field grown tomato plants by the application of extracts from various parts of *A. indica* have been reported (Goel et al., 2016). Additionally, Nahunnaro (2008) enumerated the antifungal effect of *Azadirachta indica* seed oil against yam rot pathogen (*Rhizopus stolonifer*), Joseph et al. (2008) and Nwogbaga and Utobo (2012) reported significant control of wilt and leaf spot diseases in Brinjal (*Solanum melongena*) by application of aqueous, ethanol and acetone extracts of *A. indica* leaves and seeds. The diverse findings clearly suggest that the use of *A. indica* in plant disease management has a vast future.

We observed a direct inhibitory effect of *A. indica* extract on *M. fijiensis*. Previously, it has been well documented that antimicrobial compounds are abundant in medicinal plants (Sangeetha et al., 2013; Ghosh et al., 2002; Fiori et al., 2000; Gayatri & Rajani, 2014) and these compounds could be involved in the defence of plants against pathogens. Interestingly, when two leaves on the same plant, one treated with extract and the other untreated, were inoculated with *M. fijiensis*, a disease reduction was observed only on the treated leaf. Furthermore, when the extract was soil drenched, the plants could not be protected when they were inoculated with the pathogen at 45 DAI. These observations indicate that protection was due to a direct effect and that no systemic effects were present. Indeed, several plant extracts have been reported to be capable of reducing diseases in plants through direct antimicrobial effects on the causal organisms (Guleria & Kumar, 2006; Konstantinidou-Doltsinis et al., 2006). Thousands of diverse natural products are produced by plants and many of these are involved in plant defence. The phytochemical diversity of antimicrobial compounds include terpenoids, saponins, phenolics and phenyl propanoids, pterocarpans, stilbenes, alkaloids, glucosinolates, hydrogen cyanide, indole and also elemental

sulphur, the sole inorganic compound (Vera-Guzmán et al., 2011; Snehlata et al., 2014). In this study, the phytochemical analysis of the aqueous extract of *C. zeylanicum*, *A. indica* and *C. annuum* (Table 4) showed the presence of different groups of secondary metabolites such as flavonoids, tannins, alkaloids and phenolics which are of medicinal importance. The result of our present study is in agreement with the results published by the other research groups (Prashanth & Krishnaiah, 2014; Susmitha et al., 2013).

Timing of the *A. indica* application was crucial for reduction in the *M. fijiensis* lesion number, as *A. indica* extract applied 6 DBI had no effect on lesion development, *i.e.*, this extract acted as a protectant. The production of *M. fijiensis* ascospores was reduced depending on the timing of extract application. *A. indica* extract applied 6 DBI had no effect on ascospore production. When comparing ascospore production per 3 lesions (approximately 3mm in diameter) in the infected leaf area, reductions of 36.1-65.7% (P < 0.001) were also seen and this indicates that in the lesions, the pathogen was weakened and therefore produced fewer ascospores per lesion area. This implies that *A. indica* also influenced pathogen development after penetration, although this was not confirmed using microscopy. Such an observation could have a significant epidemiological impact, as the amount of inoculum available would be reduced since ascospore is the main mode of spread of *M. fijiensis*.

To test whether induced resistance was involved in the protection observed, expression of PR-protein genes during the infection process in the susceptible banana cultivar cv. Musakala and the resistant cv. Psang awak was investigated. The resistant cultivar was included as a positive control to achieve a better insight into the expression of the target genes during an incompatible interaction. *PR-1* and *PR-3* as well as *NPR1* were differentially up-regulated in cv. Psang awak. This is in contrast to an earlier report where *PR-3* and *NPR1B* transcription was not induced in cultivated banana (GCTCV-218) in response to *Fusarium oxysporum* (Endah et al., 2008). *NPR1B* expression is related to the expression of the two PR-related genes, *PR-1* and *PR-3*, included in the study. The *non-expressor of pathogenesis-related gene 1* (*NPR1*) is an essential positive regulator of systemic acquired resistance (SAR) (Endah et al., 2008) and induced systemic resistance (ISR) (Pieters et al., 1998). Being a hemibiotrophic fungus, *M. fijiensis* acts mainly as a necrotroph at the late stages of disease progression, which was part of the timing selected for the study of the expression of the target genes. Our findings suggest that SA signalling transduction pathways, which are generally correlated with resistance against necrotrophic pathogens (Glazebrook, 2005), are active during late stages of this incompatible interaction. Unlike necrotrophic pathogens, biotrophs and hemi-biotrophs, have been reported to induce predominantly a SA-mediated pathogen response (Spoel et al., 2007; Tao et al., 2009) and SA-elicitation has been shown to induce *NPR1B* transcription (Endah et al., 2008; Yocgo, 2011). Timely recognition of the pathogen and rapid, appropriate expression of defence responses has been reported to make a key difference between susceptible and resistant plants (McDowell & Dangl, 2000; Umemura et al., 2003; Bennett et al., 2005). Black Sigatoka development in the susceptible cultivar cv. Musakala is probably because defence mechanisms were induced too late.

The activity of natural plant products on the host tissue may involve direct interaction with the pathogen or induction of host resistance (Sangeetha et al., 2013; Capdeville et al., 2002). Previously, several authors have reported the association between higher levels of chitinase and greater disease resistance against postharvest diseases. In the present study, expression of *PR-3* and *PR-1* were up-regulated early during infection in the resistant cv. Psang awak, indicating an early induction in resistant plants. The levels of the two genes were substantially increased during the late stages of infection, further indicating high induction of these genes as the pathogen switched to the necrotic stage. The expression of *NPR1* was consistent with the expression of *PR-3* and *PR-1* in cv. Psang awak plants challenge inoculated with *M. fijiensis*. To examine further whether *A. indica* could induce resistance against *M. fijiensis*, quantitative RT-PCR of the defence response genes *PR-1*, and chitinase (*PR-3*) were performed. These genes were selected because PR-proteins have previously been shown to be involved in the defence against *M. fijiensis* (Yocgo et al., 2011; Endah et al., 2008). However, none of the genes showed elevated expression when comparing plants treated with *A. indica* + *M. fijiensis* with plants treated with *M. fijiensis* alone. Collectively, our data therefore suggest that induced resistance is not responsible for the reduction in *M. fijiensis* severity by foliar application of *A. indica* extract. Of course, it is not possible to conclusively state that induced resistance is not part of the protection on the sole basis of the absence of expression of very specific defence responses, as other defence responses may be enhanced. Induced resistance as a mechanism, does not rely on the expression of specific, single defence genes. Therefore, the *A. indica*-mediated reduction in disease was most likely related to the direct inhibitory effect on the pathogen.

5. Conclusion

The finding that *A. Indica* leaf extract reduced the mycelia growth of of *M. fijiensis* implies that application of the extract should be conducted as a preventive treatment to reduce Black Sigatoka disease severity. However, *A.*

indica leaf extract may also have an impact on the epidemiological development of the disease in terms of reduced sporulation potential (ascospore formation) of the pathogen, as we observed. Ascospores are the main mode of spread of *M. fijiensis*. These issues need to be verified in field trials. Moreover, formulation of *A. Indica* extract with surfactants such as alkylbenzene sulfonates that improves coverage over an area may enhance the feasibility of the *A. indica* extract for spray applications by retaining the extract longer on the target area, slowing evaporation and inhibiting degradation by sunlight. The prospects of developing *A. Indica* extract into a commercial product are therefore promising, especially as alternative control options are anticipated to be a major part of future sustainable crop protection strategies.

Acknowledgements

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