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Induced systemic resistance using non-pathogenic micro-organisms for the control of black sigatoka in banana

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Abstract

Black sigatoka is a devastating disease of banana caused by *Mycosphaerella fijiensis.* Existing cultural and chemical strategies to control the disease have not been successful. *Pseudomonas fluorescence,* a plant growth promoting rhizobacteria, and an endophyte, *Fusarium oxysporum* (V5W2), were assessed for efficacy in controlling Black sigatoka. Two month-old disease-free banana plantlets of cvs. Mpologoma (AAA-EA) (susceptible to black sigatoka), Nfuuka (AAA-EA) (moderately tolerant) and Kayinja (ABB) (tolerant) were treated with spore suspensions of 0.5 x 10^5 and 0.5 x10^6 spores/ml of *P. fluorescens* and *F. Oxysporum,* V5W2, and tested vs. an untreated control. The plants were then challenge-inoculated with an *M. fijiensis* suspension of 5.7x10^5 mycelial fragments/ml. Black sigatoka disease was assessed every five days until 60 days after infection (DAI). Disease was assessed visually as percent infected leaf area. Results showed significant reductions of disease symptoms in *P. fluorescens*- and *F. oxysporum*-treated plants compared to the control that was only inoculated with *M. fijiensis*. For *P. fluorescens,* results on gene expression showed that a higher

expression of the Non-expressor of Pathogenesis Related gene 1B (NPR1B) was exhibited at 9 DAI than at 17 and 23 DAI. Jasmonate/Ethylene responsive genes *Hevein-like protein* (Hel) and *Allene Oxide Synthase* (AOS) were expressed at 23 DAI, with a higher expression in leaves treated with both *P.fluorescens* and *M.fijiensis.* For *F. oxysporum* (V5W2), *NPR1B* was expressed at all time points, i.e., 0, 9, 17 and 23 DAI. *Chitinase* (PR3) was also expressed on all tested time points and showed an increasing trend in expression from 9 to 23 DAI. Expression of *NPR1B* in plants pre-treated with *P. fluorescens* or *F. oxysporum* (V5W2) then challenged with *M. fijiensis,* as well as the up-regulation of the above-mentioned defense genes downstream of *NPR1B* indicate that *P. fluorescens* and *F. oxysporum* can induce resistance against Black sigatoka in banana.

Key words: AAA-EA and ABB banana genomes, *Chitinase* (PR3), *Hevein-like protein* (Hel) and *Allene Oxide Synthase* (AOS), Non-expressor of Pathogenesis Related gene 1B (NPR1B)

Introduction

The East African Highland Banana (EAHB; *Musa* spp. AAA-EA) is a crop of fundamental importance to the people in Great Lakes region of East Africa. It provides more than 25% of the carbohydrate requirements and other dietary needs, notably: fibre, potassium, phosphorus, calcium and vitamins A, B6 and C for over 80 million people (Nyombi, 2019a). Grown in more than 130 countries, bananas have an annual world production of about 145 million tonnes (Ploetz *et al.,* 2015). Uganda is the largest producer of banana and plantain in sub-Saharan Africa (SSA), followed by Rwanda, Ghana, Nigeria, and Cameroon (Dunstan, 2021). The country produces about 9.8 million tonnes, accounting for 11.2% of the total world production (Nyombi, 2019b). During the past 30 years, banana production has been declining due to various production constraints including diseases (Smale and Tushemereirwe, 2007; Sanya *et al.,* 2017; Lee, 2023). Black sigatoka (*Mycosphaerella fijiensis*) is a devastating disease that affects most highland banana clones (AAA-EA) but not the ABB group, which is known to be resistant (Tushemereirwe *et al.,* 2004; Friesen, 2016). Some AAB genome such as plantains and desert bananas are reported to be equally susceptible, with cultivar *Kayinja* of the ABB group being the only cultivar that was found to be tolerant to Black sigatoka (Kumakech *et al.,* 2017).

Numerous control strategies have been applied to manage the disease, but many of them have not been successful. Cultural practices, through removing of diseased leaves and/or pruning branches to improve air circulation have only slightly reduced the occurrence of the disease (Etebu and Young-Harry, 2011). Breeding for resistance is difficult because EAHB banana is highly ploidy thus sterile (Bennet and Arneson, 2003). Furthermore, Black sigatoka resistant materials that were acquired from

international breeding programs were disliked by the farmers due to their poor taste and lack of market (Nowakunda and Tushemereirwe, 2004; Barekye, 2009). Consequently, commercial banana growers have heavily relied on actively applying fungicides. It is reported that *Mycosphaerella fijiensis* requires the highest annual fungicide application of all pathogens globally (more than $2.5 billion) (Churchill, 2011). This heavy chemical use is unaffordable for small scale farmers not only because of its costliness and build-up of fungicide resistance in the pathogen, but also its serious health implications for consumers and banana workers with reported risks of leukemia, birth defects and male sterility (Wilson and Otsuki, 2004; Silva *et al.,* 2024). Therefore, the threat posed by Black sigatoka and the limitations in the available control measures implies that new approaches to control the disease must be sought.

An alternative control measure is biological control of the pathogen. This is an environmentally friendly method with a large potential to control diseases (Wang *et al.,* 2005; Maurya, 2020; He *et al.,* 2021). Plant growth-promoting rhizobacteria (PGPR) are bacteria, which are established well in the plant rhizosphere, and can enhance plant growth and reduce the severity of plant diseases (Gray and Smith, 2005). They are a highly promising group of organisms for control of phytopathogens (Nga *et al.,* 2010; El-Saadony *et al.,* 2022). Research has shown that PGPR can directly suppress phytopathogens through competition with the pathogens for iron via siderophores, by producing substances or antibiotics toxic to the pathogens, or by producing enzymes such as chitinases and β-1,3-glucanases that lyse fungal cell walls (Pathak *et al.,* 2017). Furthermore, they may induce resistance in the host against pathogens (Pathak *et al.,* 2017; Wang *et al.,* 2021). In several plant-PGPR combinations, colonization of roots by PGPR leads to a state of induced resistance not only in the colonized roots, but also in the whole plant. This phenomenon is designated induced systemic resistance (ISR) (Wang *et al.,* 2005; Nga *et al.,* 2010). *Pseudomonas fluorescens*, strain WCS417, *Pseudomonas fluorescens* strain FPT9601-T5 were reported to trigger ISR in Arabidopsis against *Pseudomonas syringae* pv. tomato (Wang *et al.,* 2005). In addition to PGPR, endophytic microorganisms are another promising, easily applicable, group of non-pathogenic organisms for disease control (Nga *et al.,* 2010). Fungal endophytes have been shown to protect bananas from nematodes (Waweru *et al.,* 2014; Athman, 2006) weevils, and *Fusarium oxysporum* f.sp. cubense (Foc) (Belgrove, 2007) in greenhouse and screenhouse experiments. Of the 357 fungal endophytes extracted from the roots, forty-nine showed antagonistic activity against *Fusarium oxysporum* f.sp. cubense TR4 (Foc TR4) that causes Fusarium wilt of Cavendish banana. Endophytes *Lasiodiplodia theobromae* TDC029, *Trichoderma asperellum* TDC075, *Ceratobasidium sp*. TDC037, *Ceratobasidium sp*. TDC241, and *Ceratobasidium sp*. TDC474 showed significantly high inhibitory Foc TR4 activity (79.61–99.31%) in endophyte-treated Grand Naine and GCTCV 218 plantlets

(Catambacan and Cumagun, 2021). Earlier, the two non-pathogenic micro-organisms, *Fusarium oxysporum* and *Pseudomonas fluorescens*, had shown the potential to control phytopathogens by inducing (host) disease resistance, among other modes of action (Mulero-Aparicio *et al.,* 2019; Alattas *et al.,* 2024). Induced resistance being an environmentally safe method, could be a good strategy for sustainable mitigation of Black sigatoka. In addition to the hypersensitive reaction (Lepoivre *et al.,* 2003), it is important to further understand other mechanisms used by the banana to resist *M. fijiensis* so as to enhance banana productivity and crop yields with scarce agricultural resources (Soares *et al.,* 2021). Therefore, the main objective of this research was to assess whether non-pathogenic microorganisms induce systemic resistance in banana to control *Mycosphaerella fijiensis*. Specifically, the study aimed at investigating whether i) non-pathogenic *Fusarium oxysporum* induces resistance against *Mycosphaerella fijiensis*, and ii) whether *Pseudomonas fluorescens* induces systemic resistance against *Mycosphaerella fijiensis*.

Materials and methods

Study 1: Efficacy of Pseudomonas fluorescens against Mycosphaerella fijiensis

Two month-old disease-free banana plantlets of cultivars *Mpologoma* (East African Hybrid, AAA-EA), *Nfuuka* (AAA-EA) and *Kayinja* (ABB) were obtained from the tissue culture laboratory of Makerere University Agricultural Research Institute Kabanyolo (MUARIK). The plants were grown (one per pot) in 15 cm diameter plastic pots filled with sterilized loam soil. No manure or fertilizers were applied.

Isolation of Pseudomonas fluorescens from the banana rhizosphere

For isolation of *Pseudomonas fluorescens*, 2-3 cm root pieces were cut from the banana plants grown in gardens of the research stations of Ssendusu, Kabanyolo, Kawanda and Makerere in the vicinity of Kampala City. Soil was removed from the roots after which they were rinsed with sterile water. From each root sample, 1 g tissue was taken and placed in a sterile conical flask containing 10 ml sterile distilled water and left to settle for 20 min to allow the bacteria to move from the roots into the water. The samples were serial diluted up to 10^{-9} and each dilution was plated on King's B agar (Life Technologies, India) medium and incubated at room temperature ($25 \pm 2°C$) for 72 h (Prasanna *et al.,* 2009). Distinct colonies showing yellowish green pigmentation and fluorescence at 365 nm using UV light (UVP Benchtop UV Transilluminator, BioDoc-ItTM, Cambridge, UK) were selected, purified on nutrient agar and further subjected to molecular tests (REP PCR) to identify strains of *Pseudomonas fluorescens*.

Bacterial DNA extraction

DNA extraction was carried out using the modified method of Mahuku (2004). Two millimeters sterile distilled water was added to a bacterial culture grown on King's B medium and the suspension added to micro centrifuge tubes. To each tube, 800 µl sterilized extraction buffer (50 mM NaCl, 50 mM Tris-HCl (pH 7.6), 50 mM EDTA, 5% SDS) was added and the tubes incubated at 65°C for 30 minutes. Then, 300 µl phenol and 300 µl chloroform/isoamyl alcohol were added to the tubes and mixed by vortexing for 10 seconds until an emulsion was formed. The mixture was centrifuged for 3 mins at 14,000 g to separate the phases well. The supernatant was collected and 5 µl of RNase A added and later incubated at 37°C for 1 hour. To each tube, 500 µl chloroform was added and mixed for 10 min followed by centrifugation at >10,000 g for 10 minutes. After centrifugation, the supernatant was collected in fresh 1.5 ml tubes and 0.1 volumes 3M sodium acetate and 0.7 vol isopropanol were added and mixed gently for 5 min by inverting the tubes several times and again centrifuged at >10,000 g for 10 min to precipitate the DNA. The supernatant was carefully discarded and the pellet collected. To each pellet, 500 µl 70% ethanol was added, then centrifuged at >10,000 g for 10 min. The pellet was left to dry for 1 h and later dissolved in 50 µl water for further studies. DNA quantification was done using a Nanodrop spectrophotometer (ND – 1000 UV/Vis Spectrophotometer, Nanodrop Technologies, USA) at a wavelength of 260 nm. Rep-PCR was used to determine if the bacterial culture was *Pseudomonas fluorescens*. Three families of repetitive sequences were used namely: repetitive extragenic palindromic (REP) sequence, enterobacterial repetitive intragenic consensus (ERIC) and BOX elements (Lupski, 1992). Rep-PCR fingerprinting is a highly reproducible and simple method to distinguish closely related microbial strains, to deduce phylogenetic relationships and to study their diversity in different ecosystems (de Bruijn *et al.,* 1992). PCR amplification was performed as described by Charan *et al*. (2011). A 20 µl reaction mixture containing 2 µl template, 2 µl 10x PCR buffer, 2 mM MgCl2, 0.2 mM dNTPs, 1 µl of each forward and reverse primer (Table 1), 1 U Taq polymerase (Promega Corporation) and 25 ng bacterial genomic DNA. PCR was performed in a Master Cycler (Applied Biosystems). The amplification conditions were as follows: initial denaturation at 94°C for 3 minutes, followed by 45 cycles of denaturation at 94°C for 25 seconds, annealing at 52°C for BOX AIR and ERIC primers for 1 minute and 38°C for Rep primers and extension at 72°C for 1 minute. A final extension at 72°C was carried out for 8 minutes. The PCR experiments were repeated thrice, to confirm the repeatability of the results.

Table 1. Primers used in Rep-PCR to identify *P. fluorescens*

Primer name	Primer sequence 5' - 3'		No. of bases
REP	Reverse primer	IIIICGICGICATCIGGC	18
	Forward primer	ICGICTTATCIGGCCTAC	18
ERIC	Reverse primer	TGTAAGCTCCTGGGGATTCAC	21
	Forward primer	AAGTAAGTGACTGGGGTGAGCG	22
BOXA 1R	Reverse primer	CTACGGCAAGGCGACGCTGACG	22

Only the reverse primer was given for BOX A 1R (Charan *et al*., 2011)

Testing for efficacy of Pseudomonas fluorescens for induced systemic resistance (ISR)

Preparation of bacterial inoculum

Bacterial inoculum was prepared by transferring two colonies by a loop from the bacterial culture to 100 ml nutrient broth (NB) in a 250 ml Erlenmeyer flask, which was incubated at room temperature on a rotary shaker at 130 rpm for 48 hrs (Sivaramakrishnan and Rakia, 2021). The concentration of the bacterial suspension was determined using a haemocytometer (Fisher Scientific). Nine microliters of the bacterial suspension were pipetted into each of the two counting chambers of the hemocytometer. Spores in each of the four 0.1 mm^3 corner squares were then counted and the total spores acquired by adding the number of spores in all the four corner squares. Thereafter, the average cell count per square of the four corner squares was acquired by dividing the total spore count by 4 (squares) and spore concentration, using the equation:

spores/ml = (n) x 10^4 …….....……. 1

Where: n = the average cell count per square of the four corner squares counted.
Bacterial concentration was 0.5 $x10^6$ cfu/ml and this was adjusted to also acquire a concentration of 0.5 $x10^5$ cfu/ml.

Preparation of pathogen inoculum

Infected banana and plantain leaves were collected from fungicide untreated fields at the Makerere University Agricultural Research Institute Kabanyolo (MUARIK), Uganda. Portions of the leaves with symptoms from Fouré stages 2-5 (Fouré, 1982) were selected and cut in 4 x 4 cm squares. Leaves were surface sterilized with 20% commercial sodium hypochlorite bleach plus Tween 20 (500 µL/L) for 10 minutes,

dried with sterile paper towels, placed on top of sterile filter paper soaked with sterile distilled water, and sealed inside a Petri dish with wet filter paper. Plates were incubated at room temperature for 23 days for growth of the pathogen. Pathogen cultures were grown on potato dextrose agar (PDA) (Bacterius Ltd, USA) in 9 cm Petri dishes, incubated at 25 ± 2°C (Alvarez *et al.,* 2013). Plugs, 5 mm in diameter, of *M. fijiensis* were grown in Erlenmeyer flasks containing 50 ml potato dextrose broth (PDB) (Titan Biotec Ltd, India) and incubated on a rotary shaker (150 rpm) for 3 weeks at 28°C. The mycelium was blended in water and the concentration of mycelium fragment measured using a hemocytometer. The concentration was adjusted to approximately 5.7 x 10^5 mycelial fragments/ml before inoculation. To this final concentration, 1.0% gelatine was added to increase inoculum adhesion to the banana leaves.

In-planta assay - study design and data analysis

Two month old banana plantlets were inoculated with *P. fluorescens* by drenching the soil in the pots with bacterial suspensions of either 0.5 x 10^5 cfu/ml or 0.5 x 10^6 cfu/ml (Wang *et al.,* 2005). The plants were grown and acclimatized in the greenhouse for one week with regular watering. No pesticides or fertilizers were applied. The plants were then challenge-inoculated with an *M. fijiensis* suspension of 5.7x10^5 mycelial fragments/ml by spreading it with a paint brush on the abaxial surface of the first three open leaves of banana plants pre-treated with bacteria as well as on untreated control plants (not treated with inducer). Leaves were labelled on the upper (abaxial) side and allowed to dry for 2 h. Subsequently, the plants were left to grow in a humid chamber under a relative humidity of approximately 90% and maintained for first three days by spraying continuously with water. The plants were left in this controlled environment for 60 days at a humidity of about 60-70%, as disease development was being studied. Positive controls were treated with a *M. fijiensis* mycelial suspension, but did not receive any bacterial pre-treatment. The experiment was conducted in two rounds following a complete randomized design. Disease severity was scored visually as percent leaf lamina covered by *M. fijiensis* symptoms using the Stove (1971) scale. One-way analysis of variance (ANOVA) was carried out for each parameter and means compared using Fisher's least significant differences (LSD) test at $P<0.05$. All statistical analyses were performed with SAS (version 9.2) (SAS Institute Inc., Cary, NC, USA). Disease severity rations were also used to compute Area Under Disease Progress Curve (AUDPC) (Shaner and Finney, 1977):

$$AUDPC = \Sigma i=1\ n\ [(X\ i+1 + Xi)/2][t\ i+1 - ti] \quad \ldots\ldots 2$$

Where: Xi= proportion of the host tissue damaged at i^{th} day ti= the time in days after appearance of the disease at i^{th} day, and n = the total number of observations AUDPC values were also subjected to ANOVA as described above.

Collection of tissues for gene expression studies

Leaf samples were picked from cv. *Mpologoma* banana plants at 0, 9, 17 and 23 days after inoculation (DAI) to test resistance gene expression levels (Adhikari *et al.,* 2007). Treatments were as follows: Treatment with *P. fluorescens* alone; *P. fluorescens* and *M. fijiensis*; *M. fijiensis* only (positive control); and no treatment (negative control). After harvest of samples, they were quickly frozen in liquid nitrogen and stored at -80°C until RNA extraction. RNA was extracted from the snap frozen leaves using the Zymo Reasearch Plant RNA MiniPrep kit (Epigenetics, USA). Total RNA was quantified by measuring absorbance at 260 nm using a NanoDrop spectrophotometer and by electrophoresis, running samples on 1.5% non-denaturing agarose gels. Genomic DNA was subsequently removed from all RNA samples using the DNase 1 (Thermo Fisher Scientific Inc.), incubated at 37°C for 30 min. Thermo Scientific RiboLock RNase Inhibitor was included in the reaction mixture at a concentration of 1 U/il to prevent RNA degradation.

cDNA synthesis and gene expression studies

The first strand complementary DNA (cDNA) for each of the RNA samples was synthesized using the iScript™ Select cDNA Synthesis Kit (Bio-Rad), using 1 µg total RNA in a 20 µl reaction and Oligo (dT)20 primers. The completed first strand cDNA was placed at -20°C, for use in PCR. To test whether plant defense was activated by *P. fluorescens*, expression of genes involved in signal transduction and defense were studied using qualitative PCR, the Non-expressor of Pathogenesis-Related 1 (NPR1), lipoxygenase (LOX1), allene oxidase synthase (AOS), Hevein-like protein/ PR4 (Hel), Chitinase B (Chi B), Plant defensin gene (PDF 1.2) (Paparu, 2008). The primers used are in Table 2.

Study 2: Efficacy of Fusarium oxysporum against Mycosphaerella fijiensis

Two month-old disease free banana plantlets of cultivars *Mpologoma* (AAA-EA) (susceptible to Black sigatoka), *Nfuuka* (AAA-EA) (moderately tolerant) and *Kayinja* (ABB) (tolerant) were obtained from the tissue culture laboratory of MUARIK. The plants were grown, one per pot, in 15 cm diameter plastic pots filled with sterilized loam soil. A non-pathogenic, endophytic *Fusarium oxysporum* strain (V5W2) was obtained from the International Institute of Tropical Agriculture (IITA), Kampala, Uganda, where it is usually stored in soil. This strain was earlier isolated from EAHB plants and was selected due to its demonstrated ability to induce resistance against *Radopholus similis* in banana (Paparu *et al.,* 2009).

Table 2. Primer sequences studied in cv. Mpologoma (AAA-EA) for PCR amplification of resistance genes, following inoculation with *Pseudomonas fluorescens* and challenge with *Mycosphaerella fijiensis*.

Target gene	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Expected product size (base pairs)
NPR1B	AGGTCGATTGAAGAAGGAGAAG	GATACAACAATCTTCCCCGCA	150
Hel	ATCTGCTGCAGTCAGTACGG	TGAGCTCATTGCCACAGTCG	170
Lox1	AACTTAGGGATCTGCTTCCC	CTCTATACTTGAGCAGCGTC	226
ChiB	GCTTCAGACTACTGTGAACC	TCCACCGTTAATGATGTTCG	187
PDF1.2	ACAGAAGTTGTGCGAGAA	CTGGGAAGACATAGTTGC	107
AOS	GGGGAATAAACAATGCG	ACGGCGACGTACCAAC	213
Actin	GAGAAGATACAGTGTTCTGGA	ATTACCATCGAAATATTAAAAG	231

Cultivation and preparation of F. oxysporum inoculum

Fusarium oxysporum V5W2 was sub-cultured from soil on half strength Potato Dextrose Agar (PDA) medium (19.5 g PDA and 6.6 g Agar-Agar in 1 l distilled water) and incubated for 14 days at 25°C (Paparu *et al.,* 2009). Fungal spores were harvested by adding sterilized distilled water to the culture plates and scraping off the fungal mycelial and spore growth using a sterile spatula. The spore density was estimated using a hemocytometer. The suspension was adjusted to provide a final spore concentration of 1.5×10^6 spores/ml and from here, a dilution of $1.5x10^5$ spores/ml was made with sterile distilled water. The inoculum was prepared as in study 1.

In-planta assays - study design and data analysis

Banana plants (cvs. *Kayinja, Nfuuka* and *Mpologoma*) were treated by drenching the soil with F. *Oxysporum* (strain V5W2) spore suspensions of $1.5x10^5$ and $1.5x10^6$ spores/ml (Paparu *et al.,* 2004). The control plants were drenched with distilled water. The plants were left to grow in 1 litre pots for a period of four weeks in sterilized soil. They were watered regularly and no pesticides or fertilizers were added. The plants were then challenge-inoculated with a *M. fijiensis* suspension and the experiment conducted as for *P. fluorescens*. The experiment was conducted in two rounds in a complete randomized design (CRD). The disease was assessed nine times (every five days from 15 Days After Infection (DAI)) and the final assessment was carried out at 60 DAI. Disease was assessed visually as percent infected leaf area using the Stover (1971) scale. One-way analysis of variance (ANOVA) was carried out for each parameter and means compared using Fisher's least significant differences (LSD) test at $P\leq0.05$. For gene expression studies, leaf samples of cv. *Mpologoma* were collected and the treatments conducted similarly to *P. fluorescens* as in study 1.

RNA extraction and cDNA synthesis and gene expression studies

RNA was extracted from the snap frozen leaves using the Zymo Reasearch Plant RNA MiniPrep kit as described in study 1. The first strand complementary DNA (cDNA) for each of the RNA samples was synthesized using the iScript™ Select cDNA Synthesis Kit (Bio-Rad) using primers shown in Table 3.

Table 3. Primer sequences studied in banana cultivar Mpologoma (AAA-EA) for PCR amplification of resistance genes, following inoculation with non-pathogenic *Fusarium oxysporum* and challenge with *Mycosphaerella fijiensis*.

Target gene	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Expected product size (base pairs)
NPR1B	AGGTCGATTGAAGAAGGAGAAG	GATACAACAATCTTCCCCGCA	150
PR-1	ACTACGACTACAACAGCAACAA	GTAGTTGCAGGTGATGAAGATG	150
PR-2	TACGATCCAAACAAGCCGC	ATCGAAAGGAGACGCTGG	150
PR-3	ATCTTTACCCTGGCCTCG	ATTGGCTCTGGCATCCTT	150
PR-5	CAACAACTGCGGGTTCAC	TGTTGCCGTTGAAGGAGC	150
POX	CGGTAGGATCCAAAGAAAGC	TTCAGAGCATCGGATCAAGG	150
Actin	GAGAAGATACAGTGTTCTGGA	ATTACCATCGAAATATTAAAAG	231

Microscopy of Mycosphaerella fijiensis

A 50% solution of chloral hydrate was made by mixing 125 g of the chemical with 250 ml distilled water. The chloral hydrate was dissolved in a few minutes and then was ready for use. Leaf tissue from cv. *Mpologoma* infected leaves were cut off and placed in the chloral hydrate solution in a petri dish. In order to keep the leaves under the fluid, they were covered with a piece of gauze to prevent them from floating on the surface. Clearing lasted for 2 days. The cleared leaves were then viewed under the microscope.

Results

Molecular identification of Pseudomonas fluorescens

Ten putative *P. fluorescens* isolates were subjected to Rep-PCR to confirm whether they, in fact, were *P. fluorescens.* The sizes of DNA bands generated from the PCR ranged from 100-400 base pairs (Fig. 1). Since all the bacteria were *P. fluorescens*, an isolate with a consistent band size for at least two or all the three primers in the Rep-PCR was selected for this study. Isolate 9 was selected because it had consistent band sizes (290 base pairs) for the BOX and REP primers.

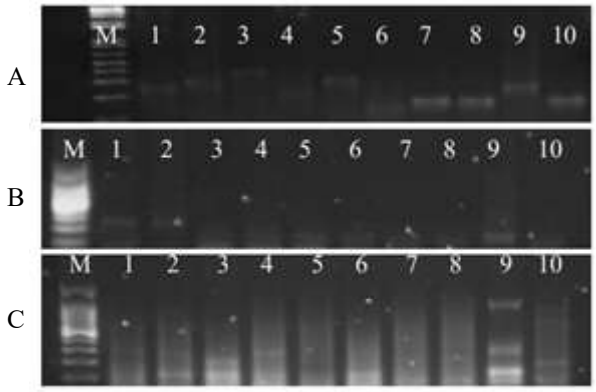

Figure 1. Picture of an agarose gel of *P. fluorescens* (A) BOX1I R; (B) ERIC; (C) REP. DNA ladder M is 100 base pairs.

Severity of Black sigatoka after inoculation with Pseudomonas fluorescens
Disease severity assessed every seven days from the first day of symptom observation to 60 days after inoculation with *M. fijiensis* generally showed a significant reduction in *P. fluorescens*-treated plants compared to the controls only inoculated with *M. fijiensis* ($P<0.05$) (Fig. 2)

Figure 2. cv. Nfuuka at 60 DAI infected with *M.fijiensis* in plants where the soil was drenched with *Pseudomonas fluorescens*: A) pre-treated with *P. fluorescens* (10^6 cfu), showing brown lesions of Black sigatoka with thin, dark edges and small water-soaked halos; B) pre-treated with *P. fluorescens* (10^5 cfu) showing larger brown lesions and larger water-soaked halos. C) Control with no pre-treatment with *P. Fluorescens.*

In cv. *Nfuuka* (Fig. 3C), disease symptoms were first observed at 25 days after inoculation (DAI) yet for cv. *Kayinja* (Fig. 3A), considered tolerant to Black sigatoka, there were no disease symptoms observed at this time. For *Nfuuka*, there was a

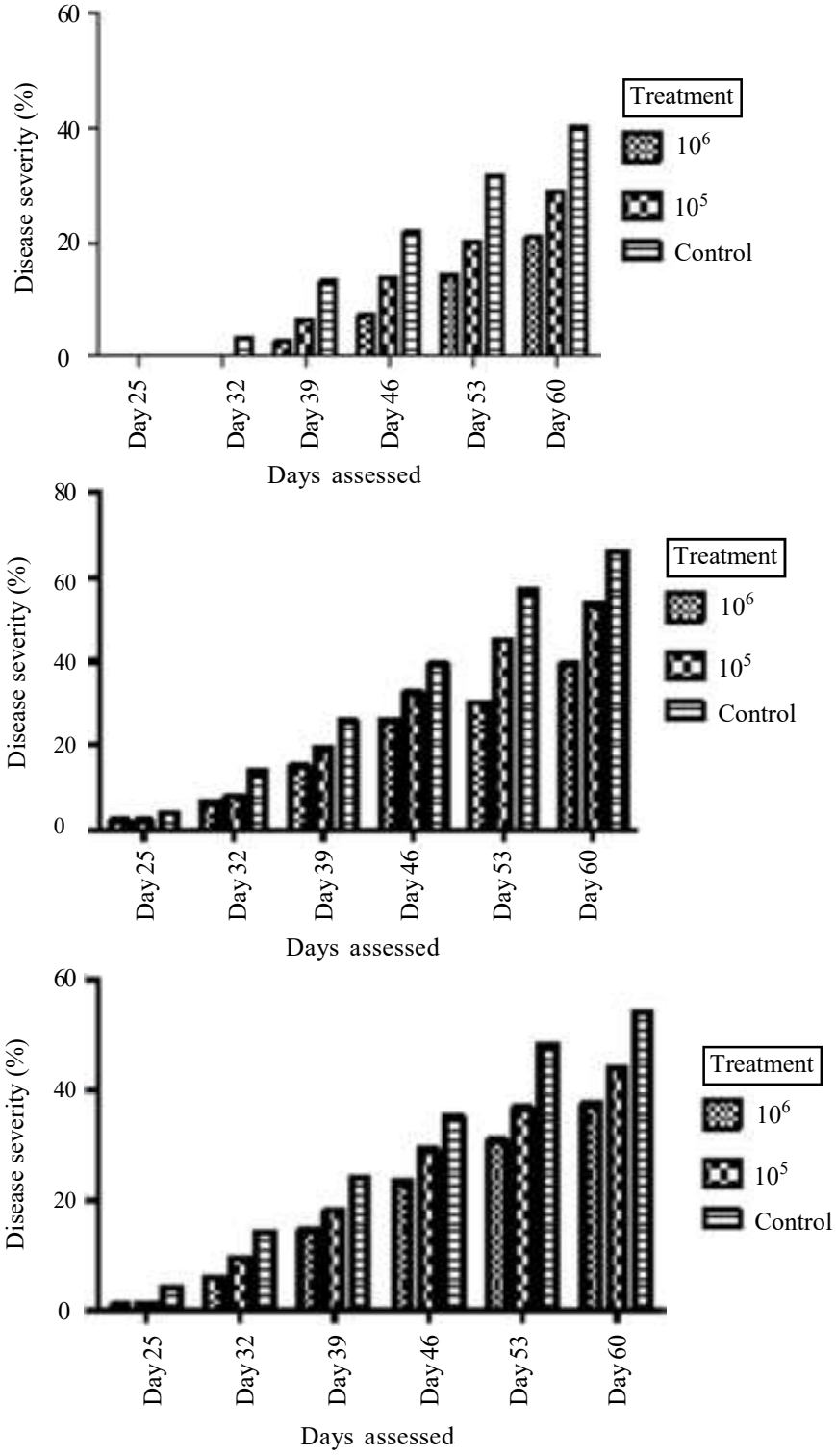

Figure 3. Average weekly severity assessments of Black sigatoka in (A) Cv. Kayinja; (B) Cv. Mpologoma; and (C) Cv. Nfuuka after pre-treatment with *P. fluorescens* and challenge inoculation with *M. fijiensis.*

significant difference (P = 0.0093) between the control and the *Pseudomonas* pre-treated plants. By 25 DAI, symptoms appeared as minute reddish specks on the lower leaf surfaces (Stage 1) and at 32 DAI, these were increasing to brown streaks on the upper leaf surface (Stage 3). All in all, the number and size of specks/streaks on the *Pseudomonas* pre-treated plants was less than on the controls (not inducer-treated).

The overall severity of Black sigatoka from the start to the end of the experiment is shown in the AUDPC plotted against treatments, which showed that the *Pseudomonas* pretreated bananas were less severely infected than the controls (Fig. 4). The highest *Pseudomonas* concentration of 0.5×10^6 cfu/ml gave the lowest disease severities in all cultivars studied, with a significant difference at P = 0.047 ($F_{df\,14;\,17} = 20.71$), calculated using AUDPC values.

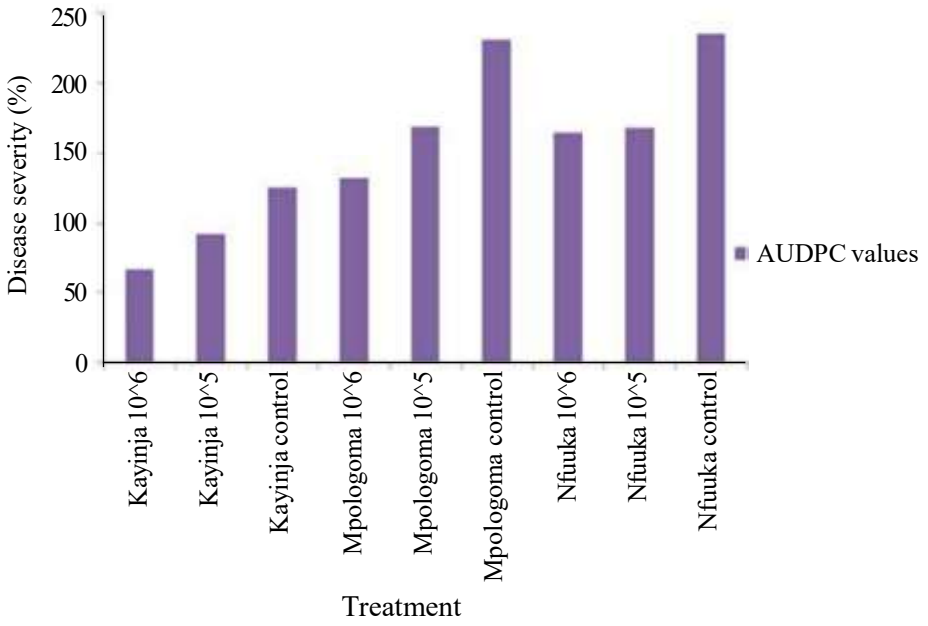

Figure 4. Disease intensity (AUDPC) of Black sigatoka in three banana cultivars by 50 DAI after pre-treatment with *P. fluorescens* followed by inoculation with *M. fijiensis*.

Expression analysis of genes induced by P. fluorescens against M. fijiensis

Cultivar *Mpologoma* was used for this study as a representative of susceptible East African Highland Banana cultivars used in this work. Plant responses to these treatments were investigated systemically, by analysing accumulation of defense-related genes at 0, 9, 17 and 23 DAI. Genes ChiB, Pdf1.2 and Lox 1 were not induced during the study period (data not shown). However, known defense genes AOS

(Allene oxide synthase), NPR1B (Non-expressor of Pathogenesis Related gene 1B), and ethylene-inducible gene Hel (Hevein-like protein) were expressed at variable points (Fig. 5). NPR1B was expressed at 9 DAI in *M. fijiensis*-treated plants, had a weak expression in *Pseudomonas*-treated plants but to a higher extent in the sample treated with both *P. fluorescens* and *M. fijiensis,* compared to the plants only treated with *Pseudomonas* (product seen at 150 bp). At 17 DAI, NPR1B was only slightly expressed in *Pseudomonas*-treated plants, and at 23 DAI, this gene was neither expressed in *Pseudomonas*-treated plants nor in the sample treated with both inducer and pathogen. There was, however, a slight expression in the *M. fijiensis* treated plants. Similarly, *AOS* was only expressed at 23 DAI with a product size of 213 bp, in plants treated with both the pathogen and inducer. *Hel* (product size 170 bp) was also expressed at a low level at 23 DAI in the sample inoculated with *P. fluorescens* and those treated with both *P. fluorescens* and *M. fijiensis*.

Severity of Black sigatoka after inoculation with Fusarium oxysporum, V5W2

Overall, severity of Black sigatoka differed significantly between the controls and the *Fusarium*-treated plants ($P < 0.05$). Mean comparisons revealed that cv. *Mpologoma* was most severely attacked by the disease. Kayinja, a cultivar known to be tolerant to Black sigatoka showed late disease initiation and slow symptom development

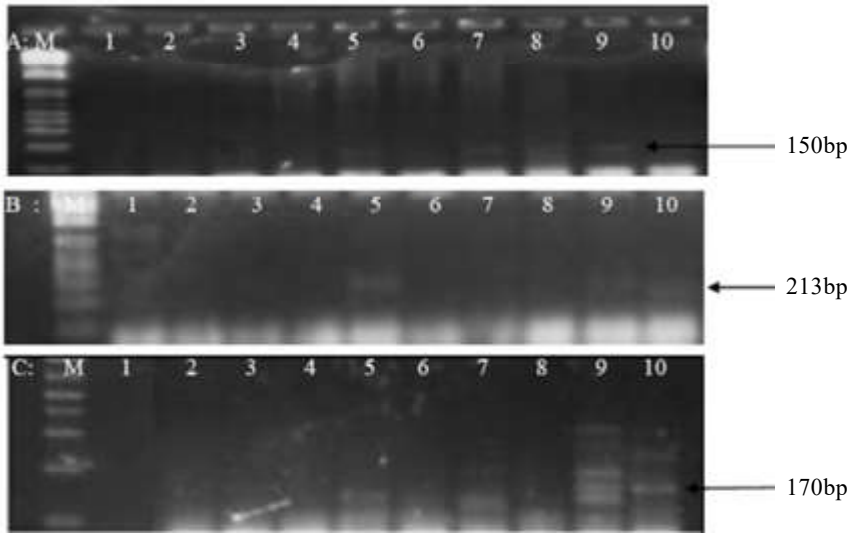

Figure 5. Amplicons of (A) NPR1B; (B): AOS and (C) Hel amplified from banana after treatment of banana roots with *P. fluorescens* and challenge inoculation with *M.fijiensis*. Lane 1: day 0 (negative control); Lanes 2-4 (9 DAI); Lanes 5-7 (17 DAI); Lanes 8-10 (23 DAI). Lane 1 was uninoculated (negative control); Lanes 2,5 and 8: only inoculated with *M.fijiensis* (positive control); Lanes 3,6 and 9: treated with *P.fluorescens*; Lanes 4,7 and 10: treated with *P.fluorescens* and inoculated with *M.fijiensis*. DNA Ladder M is 100 base pairs.

compared to the other two cultivars (Fig. 6). Mpologoma also showed significant differences between the controls and the plants treated with *F. oxysporum* (Fig. 7). However, there were no significant differences in disease severity between the plants treated with the two *F.oxysporum* concentrations (10^6 and 10^5). *Nfuuka* cultivar showed that differences in disease severities were significant on days 50, 55 of symptom development (P = 0.0005, P = 0.0010, respectively), and also showed significant differences between the control plants and the plants pre-treated with both the *F. oxysporum* concentration of 10^6 and 10^5 on day 20, 35, 40, 45, 50, 55 (P = 0.02, P = 0.03, P = 0.01, P = 0.003, P = 0.01, P = 0.01, respectively) (Fig. 7).

A B C

Figure 6. Severity of Black sigatoka on cv. Kayinja at 45 DAI with *M. fijiensis:* A) not treated with *F. oxysporum* (Control); B) pre-treated with *F. oxysporum* at $5x10^5$ cfu/ml; C) pre-treated with *F. oxysporum* at $5x10^6$ cfu/ml.

The overall severity of Black sigatoka by the end of the experiment is shown in the AUDPC values plotted against treatments (Fig. 8). *Fusarium*-pretreated bananas were less severely infected than the controls (P = 0.003; $F_{df\,8;\,17} = 8.7$).

Expression analysis of resistance genes induced by F. oxysporum against M. fijiensis

The study done to determine whether *F. oxysporum* (V5W2) could suppress development of Black sigatoka by induction of resistance, showed that only (NPR1B and PR-3) of the six genes studied were expressed (Fig. 9). Expression of NPR1B (product 150 bp) was low at 9 DAI in sample 2, 3 and 4 (Fig. 9A). At 17 DAI, there was also a faint expression of NPR1B in samples 5, 6, 7 (Fig. 9A). Higher levels of expression were seen at 23 DAI in samples 8, 9 and 10 (treated as above). Faint expression of NPR1B was also seen in the leaf tissue by day 0. This study also showed expression of PR-3 (product at 150 bp) at all time points tested. Highest

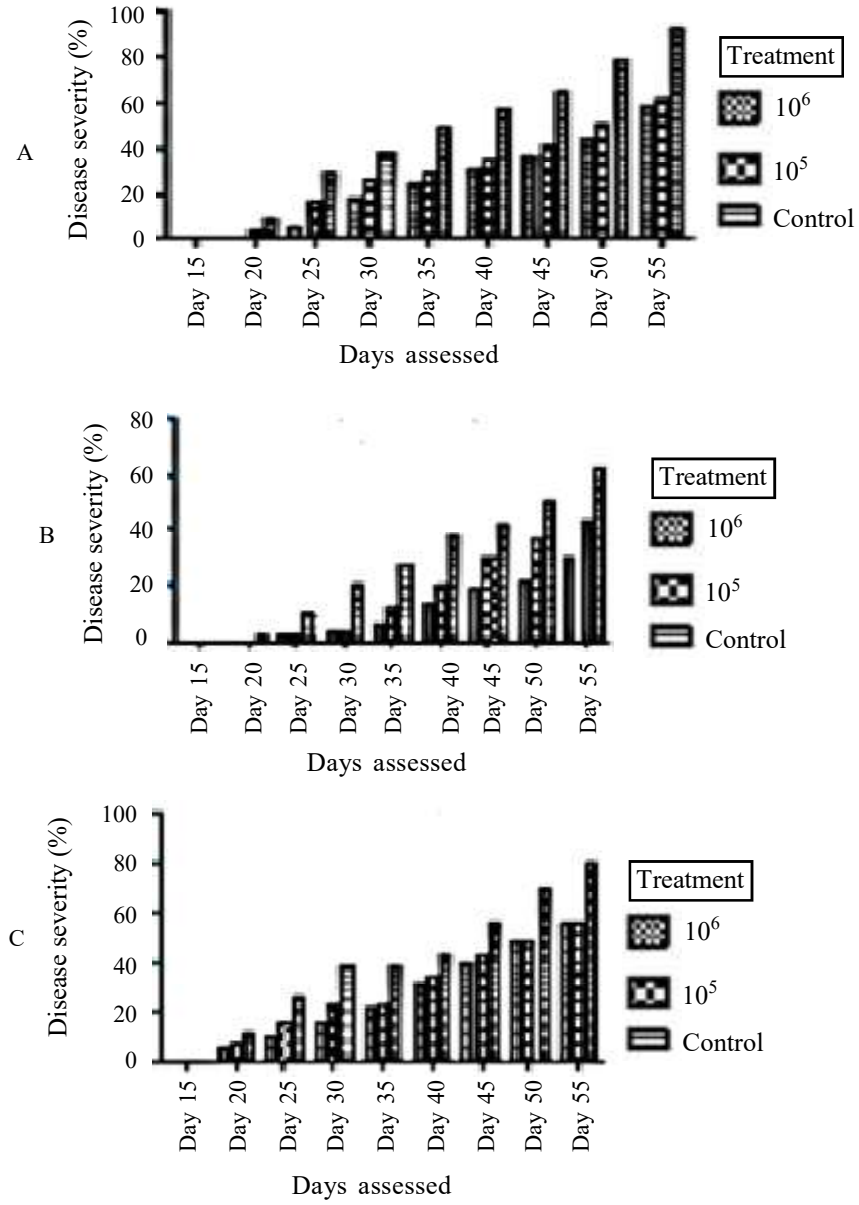

Figure 7. A) Average weekly disease severity assessed in cv. Mpologoma; B) cv. Kayinja; C) cv. Nfuuka after soil drenching with *F.oxysporum* V5W2 and inoculation with *M.fijiensis*.

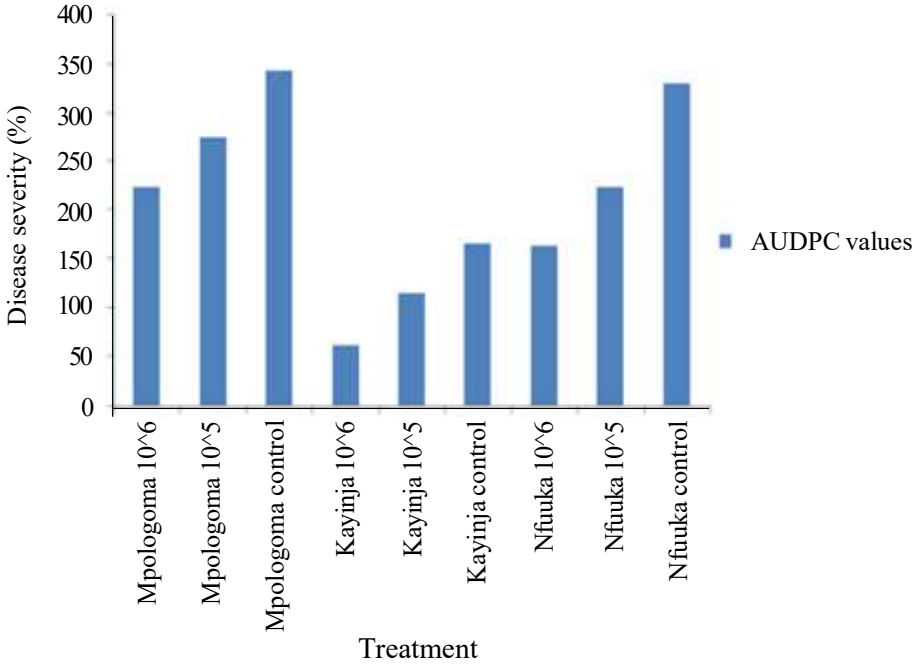

Figure 8. Severity ratings of Black sigatoka in three banana cultivars at 60 DAI with *M. fijiensis* after pre-treatment with *F. oxysporum.*

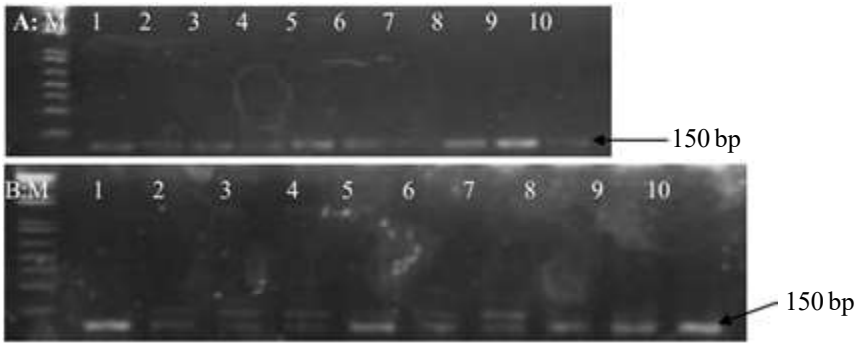

Figure 9. Cv. Mpologoma. Amplicons of the genes: (A) NPR1B, and (B) PR-3 amplified from banana leaves after treatment of banana roots with *F. oxysporum* and inoculation of the leaves with *M. fijiensis*. Lane 1: day 0 (negative control); Lanes 2-4: 9 DAI; Lanes 5- 7: 17 DAI; Lanes 8-10: 23 DAI. Lane 1 was uninoculated (negative control); Lanes 2, 5 and 8: only inoculated with *M. fijiensis* (positive control); Lanes 3, 6 and 9: treated only with *F. oxysporum*; Lanes 4, 7 and 10: pre-treated with *F. oxysporum* and inoculated with *M. fijiensis*. DNA ladder M is 100 base pairs.

expression of this gene was observed at 23 DAI in samples only inoculated with *M. fijiensis* (lane 8), lane 9 (only pre-treated with *F. oxysporum*) and 10 (treated with both *F. oxysporum* and *M. fijiensis*) (Fig. 9B). The trend showed that PR-3 expression was low at 9 DAI (samples 2, 3, 4) and increased at the subsequent time points studied.

Discussion

The aim of this study was to determine whether non-pathogenic microorganisms can protect the banana plant against *Mycosphaerella fijiensis* and whether the reductions in disease were due to induced resistance. Generally, from the results of the two studies, the indication was that *Pseudomonas fluorescens* and *Fusarium oxysporum* (V5W2) can reduce the rate of Black sigatoka development in the banana cultivars studied. Plants treated with only *M. fijiensis* had higher disease severity compared to those pre-treated with the inducers. Further inquiry on the induction of resistance by these inducers against Black sigatoka revealed that pre-treatment with *P. fluorescens* and *F. oxysporum* elicited expression of defense-related genes, and this could be the mechanism behind the reduction in disease expression. Additionally, the overall disease severity shown by the AUDPC values plotted against their respective treatments showed lower disease severities in the inducer–treated plants than the untreated plants (controls). This signified that overall, the inducers slowed down Black sigatoka progression compared to the controls.

As expected, AUDPC values of cv. Kayinja were lower than for cv. Nfuuka and Mpologoma, and this could be because of the difference in the genome of the cultivars. Kayinja has the ABB genome whereas the latter two cultivars have the AAA genome. Earlier research shows that ABB cultivars have tolerance/resistance to Black sigatoka (Karamura, 1999). It was also reported that cv. Kayinja and cv. Kivuvu, both ABB were less damaged by leaf spots Black and Yellow Sigatoka, and Cladosporium freckle (Tushemereirwe *et al.,* 1993).

Higher concentrations of the inducers suppressed the disease better than lower concentrations. For example, at 53 DAI, mean disease severity of cv. *Kayinja* treated with $1.5x10^6$ cfu/ml of *P. fluorescens* and subsequently challenged with *M. fijiensis* was 14%, whereas that of 1.5×10^5 cfu/ml *P. fluorescens* was 20%. Haggag and Abo El Soud (2012), also found that high concentrations of *P. fluorescens* in vitro had higher antifungal activity against *Botrytis cinerea* than low concentrations. The higher concentrations of *P. fluorescence* and *F. oxysporum* used in the study had higher antifungal activity against Black sigatoka, and studies to establish most virulent dosage would further inform science.

Induced resistance conditioned by treatment with Pseudomonas fluorescens

Pre-treatment with *P. fluorescence* provided an appreciable level protection against *M. fijiensis*. Disease progress as estimated using AUDPC was lower in *P. fluorescens* primed plants than the non-primed plants. These results corroborate other studies that show that *P. fluorescens* is efficacious for reducing disease development (Hol *et al.,* 2013). For example, in carnations, application of *Pseudomonas* sp. strain WCS 417r protected plants systemically against *Fusarium* wilt caused by *Fusarium oxysporum* f.sp. *dianthi* (Van Peer *et al.,* 1991). Other defense related studies in banana have been shown against Fusarium wilt (Li *et al.,* 2017; Sun *et al.,* 2019) and Black sigatoka (Borah *et al.,* 2022) where defense genes were elicited against the diseases. Similarly, *Pseudomonas fluorescens* CHA0 and *Pseudomonas aeruginosa* 7NSK2 induced resistance in grapevine against *Botrytis cinerea* by activating an oxidative burst and phytoalexin buildup in grape cells (Verhagen *et al.,* 2010). *Pseudomonas fluorescens* WCS374r also triggered ISR in rice (*Oryza sativa*) against the leaf blast pathogen, *Magnaporthe oryzae* (De Vleesschauwer *et al.,* 2008).

Molecular studies on the expression of the defense-related genes used in the current work revealed that the genes *AOS (Allene oxide synthase)*, *NPR1B* (Non-expressor of Pathogenesis Related gene 1B), and ethylene-inducible gene *Hel* (normally considered to be involved in ISR) were expressed when *P. fluorescens* was used as an inducer. NPR1 is a defense regulatory protein whose role is to activate responses implicated in plant defense (Pieterse *et al.,* 1998; Van Wees *et al.,* 1999). In this study, since *NPR1B* was expressed at day 0 in uninfected samples, it could show the possibility that this gene is constitutively expressed. In recent studies, *NPR1* has been reported to be constitutively expressed (Le Henanff *et al.,* 2009). In *Pseudomonas*-treated plants, *NPR1B* was expressed by 9 DAI, and also there were increased levels of the gene's expression after challenge inoculation with *M. fijiensis*. Inoculation with *M. fijiensis* alone probably activated NPR1 expression and may thus confound interpretation of these results. *NPR1B* was also induced at 9 DAI, on application of *P. fluorescens* alone, perhaps as an 'early warning' system, which resulted in a stronger expression of the gene following challenge inoculation with *M. fijiensis*. This early warning or priming is characterized by light expression of resistance markers after initial elicitation, but a strong expression of resistance after pest or pathogen attack (Paparu *et al.,* 2009). This state has been described as advantageous to the plant since it reduces fitness costs than the direct induction of defense (Van Hulten *et al.,* 2006; Conrath *et al.,* 2015). Examples of priming in plant defense was shown when direct induction of defense responses by high doses of either â-aminobutyric acid (BABA) or benzo (1,2,3) thiadiazole-carbothioic-acid-S-methylester (BTH) strongly affected both plant growth and seed production, thus reducing plant fitness (Van Hulten *et al.,* 2006). This early warning was also reported

in carnation (*Dianthus caryophylus*) following colonisation by *P. fluorescens* WCS417r where increased phytoalexin production was observed in plants challenged with *F. oxysporum dianthi* (van Peer *et al.,* 1990)

In this study, the ethylene-inducible gene *Hel*, was expressed at 23 DAI, mildly in *Pseudomonas*-treated plants and strongly after challenge inoculation with *M. fijiensis.* Previous studies have shown that *Hel* genes have RNase activities and contain a conserved N-terminal cysteine-rich chitin-binding domain, which further supports their antifungal activity in plants (N. Wang *et al.,* 2011). The role of *Hel* genes in the suppression of Black sigatoka could therefore have been to weaken the cell wall of *M. fijiensis* by binding to the chitin in the pathogen cell wall. Additionally, *Hel* could have suppressed Black sigatoka through its RNase hydrolysing the RNA of *M. fijiensis*, thus reducing fungal development. This aligns with findings that exogenous application of RNase activity in the extracellular space of tobacco leaves led to up to 90% reduction in symptom development of tobacco black shank disease (caused by *Phytophthora parasitica* var. *nicotianae*) (Caliana *et al.,* 1997). This study also showed expression of the jasmonic acid (JA)/ ethylene responsive gene *Allene oxide synthase* (*AOS*) at 23 DAI. *AOS*, being the first enzyme in the branch pathway leading to the biosynthesis of JA (; Sivasankar *et al.,* 2000; Feussner and Wasternack, 2002) indicates the possibility of *P. fluorescens* to activate the JA pathway against *M. fijiensis*. Thus, *AOS* catalyses the first step in the biosynthesis of jasmonic acid from lipoxygenase-derived hydroperoxides of free fatty acids and therefore permits direct access to the modulation of other signal compounds (Yan *et al.,* 2018; Sivasankar *et al.,* 2000). Earlier studies have shown that over-expression of flax *AOS* under the regulation of the cauliflower mosaic virus 35S promoter in transgenic potato plants led to an increase in the endogenous level of JA (Harms *et al.,* 1995). Transgenic plants had six- to 12-fold higher levels of JA than the non-transformed plants.

Induced resistance conditioned by treatment with Fusarium oxysporum

The study showed evidence of suppression of infection by *M. fijiensis* by treatment with *Fusarium oxysporum.* Further inquiry into the mechanism of disease control against Black sigatoka revealed that indeed this endophyte is capable of eliciting induced resistance against Black sigatoka disease. Molecular studies have showed that SAR defense related genes NPR1B and PR3 are up-regulated systemically in the plant during disease development (Zhang *et al.,* 2018; He *et al.,* 2023). In this study, molecular studies using endophytic *F. oxysporum* V5W2 confirmed that the defense-related genes *NPR1B* and *PR-3* were up-regulated systemically in the plant during disease development. *NPR1B* is a jasmonic acid/salicylic acid inducible gene. Up-regulation of this gene could indicate that this inducer elicits defense responses against Black sigatoka. The gene was expressed at all tested time points (0, 9, 17

and 23 DAI) and in all the samples treated with only *F. oxysporum*, with only *M. fijiensis,* and in all those treated with both *F. oxysporum* and *M. fijiensis*. Its expression at 0 DAI showed that it was constitutively expressed in the plants which were neither infected with *M. fijiensis* nor pre-treated with *F. oxysporum*. Expression of this gene was highest at 9 DAI, whereas on the subsequent days, it was weakly expressed in the plants inoculated with *F. oxysporum* and *M. fijiensis* compared to those treated with only *F. oxysporum*. This was also the case in the *Pseudomonas* experiment, where expression of *NPR1B* was at its highest at 9 DAI. Earlier studies have shown that NPR1 monomer triggers gene transcription only transiently, and after which it may be removed and replaced by the next monomer that again pulse activates gene transcription (Spoel, 2006). This is crucial for balancing the plant's defense response with normal growth processes. This could support the current results in which *NPR1B* was strongly expressed in the initial pathogen stress days, that is by day 9, and reduced in the subsequent days. NPR1 exists as an oligomer created by disulfide connections between molecules. NPR1 is reduced to a monomeric form upon SAR induction due to a biphasic shift in cellular reduction potential. Gene expression is activated when monomeric NPR1 builds up in the nucleus. Defense gene expression is prevented by inhibiting NPR1 decrease (Mou *et al.,* 2003; Backer *et al.,* 2023).

Therefore, activation of *NPR*1, leads to the formation of NPR1 monomers and subsequently, activation of PR-gene expression (Dong, 2004; Spoel *et al.,* 2006). Downstream of *NPR1B* in this study was the up-regulation of a *chitinase* (*PR-3*). This gene was expressed at 23 DAI in samples inoculated with *M. fijiensis* alone, those treated with *F. oxysporum* alone, and increasingly highly expressed in plants treated with *F. oxysporum* and *M. fijiensis. PR-3* expression exerts hydrolytic action on pathogen cell walls that contain chitin (Van Loon, 1997) weakening pathogen (fungi) cell walls. Up-regulation of PR3 could therefore have weakened the cell walls of *M. fijiensis* through its hydrolytic action. Earlier studies have shown that chitinases exhibit pronounced antifungal activity and plants over expressing chitinase show decreased susceptibility to infection by fungi with chitin containing cell walls (Heil and Bostock, 2002). In a related study, Paparu et al. (2009) reported an increase in the up-regulation of *PR-3* by 53% when banana cv. Kayinja was treated with *F. oxysporum* and challenged with the nematode *Radopholus similis*, as compared with endophyte inoculated and nematode non-challenged plants.

On the other hand, *M. fijiensis* is reported to possess avirulence genes that are secreted during infection to protect its fungal cell walls against hydrolysis by plant chitinases through binding to chitin (Stergiopoulos *et al.,* 2010). According to earlier studies, the genomic sequence of *M. fijiensis* has an effector protein, MfAvr4 (Stergiopoulos *et al.,* 2010). MfAvr4 is a homologue of *Cladosporium fulvum* (a

non-obligate biotrophic fungus that causes leaf mould of tomato), which contains a functional chitin-binding domain. Therefore, MfAvr4, may protects fungal cell walls against hydrolysis by basic plant chitinases, which could have come into play to partly protect the fungal cell wall.

The chitinase binding genes (*Chitinases* and *Hel*), and the other genes activated by *F. oxysporum* and *P. fluorescens* in this study may only be a few of the defense responses activated by these inducers in induced resistance against Black sigatoka. It is possible that there are other important defense reactions that could have been involved in the reduced development of Black sigatoka in the banana that were not captured in this study. This study used specific primer sets that could only detect the genes included in the panel, thus potentially missing unconsidered defense genes. Therefore, more research on the defense responses involved in controlling development of Black sigatoka disease could be conducted. This study confirms that the two biocontrol agents can be used in the management of Black sigatoka disease in banana and recommends using technique in integration with cultural measures.

Conclusion

Pretreatment of banana plants with non-pathogenic *Pseudomonas fluorescens* and *Fusarium oxysporum V5W2* prior to inoculation with *M. fijiensis* showed significant reductions in progression of Black Sigatoka symptoms when compared to the untreated control, with higher doses producing better suppression. Treated plants expressed defense related genes (*AOS*, Hel, PR-3) downstream of NPR1, a defense regulatory protein that activates plant defense responses, demonstrating an induction of resistance against Black sigatoka in banana. This study provides a basis for further exploration of this environmentally friendly method for sustainable management of Black Sigatoka on banana.

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