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SPECIAL ISSUE ARTICLE

Effect of Mycovirus Infection on the Hydrolytic Enzymes Activity of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4, the Causal Agent of Banana Fusarium Wilt

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ABSTRACT

Fusarium oxysporum f.sp cubense Tropical Race 4 also known as *Fusarium odoratissimum* (FO) is a fungal pathogen affecting banana (*Musa spp.*). It secretes plant cell-wall degrading enzymes (PCWDEs) to penetrate and colonize roots, resulting in wilting symptoms and host death. Mycovirus is a virus that infects fungus. It was discovered that mycovirus could be a biocontrol agent by causing hypovirulence in fungal pathogens, a typical symptom of suppressed pathogenicity of an infected host. Hypovirulence was also associated to reduced PCWDE activity in several plant fungal pathogens. To date, no hypovirulent mycovirus was reported in FO. Hence, this study was conducted to characterize a specific FO strain P2S isolate, co-infected by FO mycobunyavirales-like virus 1 (FoMYV1) and FO Unclassified RNA virus 1 (FoURV1), on its pathogenicity and production of PCWDEs in comparison to uninfected and virulent POH27 and cured CP2S isolates. Result showed that FoMYV1 and FoURV1 reduced disease severity (45.0 ± 12.6%) in P2S isolate compared to POH27 (90.0 ± 5.8%) and CP2S (90.0 ± 5.8%) isolates. Furthermore, co-infection of mycovirus and reduced disease severity were likely associated to the downregulation of PCWDE production such as exoglucanase, xylanase and protease. This is the first study suggesting the possible hypovirulence mechanism induced by the co-infection of mycovirus in FO.

Keywords: FoMYV1, FoURV1, Hypovirulent, Mycovirus, Plant cell wall degrading enzymes

Introduction

Fusarium odoratissimum (FO), formally known as *Fusarium oxysporum f. sp. cubense* tropical race 4, is a soilborne fungal pathogen affecting the banana (*Musa spp*.) production worldwide.[1–3] FO infects the banana through wounded roots and further releases hydrolytic enzymes to break down host cell walls. FO has been a bane in the banana industry that has contributed to an annual loss of 388.4 million USD in South-east Asia and 138 million AUD in the Oceania continent.[4] Observable field symptoms include

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flaccid stems, chlorotic leaves, and pseudostem splitting, leading to poor growth and death of the host.[1]

The infection of FO begins with mycelia penetration into the banana roots, followed by colonization and spreading to the vascular tissues. A successful penetration requires the production of various plant cell-wall degrading enzymes (PCWDEs) by FO to breach the first line of the plant host defense – the cell wall. Commonly reported fungal CDWEs include a combination of cellulase, protease, pectinase, xylanase, endoglucanase, and exoglucanase.[5–8] FO depolymerize components of plant cell walls, ranging from cellulose, xylan, pectin, polygalacturonic acid, and extensins.[7–9]

Mycovirus is a virus that infects fungi. The first discovery of mycovirus was reported in *Agaricus bisporus*. The infected mushroom exhibited symptoms of deformed fruiting body, retarded growth and early maturation, resulting in yield loss.[10] Mycovirus was known to transmit mostly through intracellular routes such as cell division, sporulation, and hyphal anastomosis, as mycovirus lacks extracellular route disease transmission and movement proteins. Up to 70% genome of mycovirus is known to be double-stranded RNA, while the remaining 30% consists of positive and negative single-stranded RNA.[11] Generally, most mycoviruses cause asymptomatic symptoms in their host, but there are some strains that could either enhance (hypervirulence) or reduce the pathogenicity (hypovirulence) of fungal pathogens.[4] Fungal hypovirulence is a desirable biocontrol trait in which the infected host becomes less pathogenic compared to the uninfected wild type. In addition, previous studies have reported that fungal hosts with hypovirulence symptoms showed changes in transcriptome and proteome, indicating that mycovirus regulated host metabolism to their own advantage.[12,13]

The mycovirus *Cryphonectria parasitica* Hypovirus 1, CHV1 infecting *Cryphonecteria parasitica* altered the host transcriptome and proteome by downregulating the production of PCWDEs, biosynthesis of secondary metabolites and antioxidant activity.[14] As these metabolic activities were involved in the pathogenicity of *C. parasitica*, the disease severity caused by the infected isolates was markedly lower compared to the uninfected strains.[14,15] Furthermore, the mycovirus *Sclerotia sclerotiorum* hypovirulence-associated DNA virus 1, SsHADV1, infecting *Sclerotia sclerotiorum* downregulated the production of PCWDEs and oxalic acid metabolism. Oxalic acid was found to be crucial in the pathogenicity of *S. sclerotium*.[16] *Bipolaris maydis* partitivirus 36 (BmPV36), which infects *B. maydis*, has also affected host gene expression by downregulating mycotoxin production and PCWDE metabolism.[17] These studies clearly demonstrated that downregulation of PCDWEs production in mycovirus-infected fungi was associated with the reduction of host pathogenicity.

Both biological and chemical control methods were used to control FO. Satisfactory control of FO was achieved by applying fungicides such as captan and mono-alkyl lipophilic cations, but the potential risks of affecting soil microbiome and chemical residue were still of concern.[18] To address the limitations of chemical control, biological control is an eco-friendly alternative. Antagonistic microbes or biocontrol agents (BCAs) such as *Piriformospore indica and Streptomyces morookaensis* reduced the incidence of *Fusarium* wilt in banana plants, enhanced the beneficial rhizosphere microbiome, and improved soil physicochemical properties.[19] Recently, hypovirulent mycoviruses have gained interest as potential BCA due to their ability to suppress or convert plant pathogenic fungi into non-pathogenic strains.[20,21] Though so, there are only two studies that reported the presence of mycovirus in FO, but no hypovirulent strains were identified and characterized.[22,23] Thus, this is the first study that aimed to characterize the hypovirulence mechanism of two mycovirus co-infecting a FO isolate (P2S) from Johor state, Malaysia, by comparing the PCWDE activities and pathogenicity of mycovirus-cured CP2S and virulent POH27 isolates.

Material and methods

Fungal pathogen cultivation

Three FO isolates – P2S (from the Johor state, Malaysia), CP2S (a mycovirus-cured P2S isolate) and POH27 (from the Perak state, Malaysia) were used in this study. The CP2S strain was obtained by culturing in potato dextrose agar (PDA) (Liofilchem®, Italy) supplemented with 100 μg/mL ribavirin through 5 subculture passages. The fungal isolates, stored on filter paper, were provided by the Department of Agriculture and Food Science at Universiti Tunku Abdul Rahman. The isolates were revived on PDA and incubated at 25°C. All isolates were subsequently subcultured to fresh PDA every 7 days.

FO isolates grown in PDA were further inoculated into a 100 mL potato dextrose broth, PDB (Sisco Research Laboratories Pvt. Ltd), and incubated on a shaking incubator, at 25°C for 7 days to obtain mycelial mat for total RNA extraction.

Total RNA extraction

The total RNA was extracted from fresh mycelia mat according to a previous study with modifications.[24] A total of 10 g of fresh mycelia was blotted dry on filter paper and homogenized using liquid

Table 1. Primer sequence used for mycovirus detection for FO isolates.

Mycovirus	Sequence of Primers	Amplicon size (bp)
FoURV1	**Forward**: 5'-CACGTTTAAGGTGCCATGAGAG-3' **Reverse**: 5'-CGCACGCCAGGACTCCTCGTGA-3'	~175 bp
FoMYV1	**Forward**: 5'-CTGTTGGGCTCAGTTGCTTT-3' **Reverse**: 5'-TCGCACAGTATCCCGAATGA-3'	~175 bp

nitrogen. A volume of 15 mL of lysis buffer (0.05 M EDTA, pH 8.0, 0.1 M Tris-HCl, pH 8.0, and 0.25 M NaCl), 1 mL of 10% (w/v) SDS, and 2 mL of β-mercaptoethanol were added to the ground mycelia and incubated for 5 mins. An equal volume of phenol citrate buffer saturated (pH 4.2 $\pm$0.1), chloroform, and isoamyl alcohol (P: C: I) (25:24:1) was added, mixed, and incubated for 15 mins at room temperature. The mixture was centrifuged at 12000 $\times$ g, 4°C for 10 mins, and the supernatant was separated into a new sterile tube. An equal volume of PCI was added to the supernatant and mixed well before centrifuging again at 12000 $\times$ g, 4°C. The supernatant was separated, followed by the addition of 2.5 v of ethanol. The sample was incubated overnight at –80°C.

The mixture was centrifuged at 12000 $\times$ g, 4°C, and the supernatant was discarded. The RNA pellet was washed twice with 70% (v/v) ethanol. The pellet was resuspended with 1 mL of TE buffer. DNA content was removed with DNAse I (1U/ μL) with 10X reaction buffer containing $MgCl_2$ at 37°C for 15 mins. An equal volume of PCI was used to remove DNAse, mixed and centrifuged at 12000 $\times$ g, 4°C for 5 mins. The pellet was precipitated with 2.5 v ethanol for 10 mins and centrifuged again at the same conditions as above. Then the samples were then washed with 100 μL of 70% (v/v) ethanol and centrifuged at the same condition as above before resuspending with 50 μL of RNase free water. The quantity and quality of RNA extracted were determined using NanoDrop™ 2000/2000C spectrophotometer to ensure the quantity (>700 ng/μL) and quality of absorbance ratio (Absorbance at 260 nm/230 nm: 2.00–2.20; Absorbance at 260 nm/ 280 nm: 1.8–2.20) were within optimal range before storing in –80°C for subsequent use.

cDNA synthesis and reverse transcription PCR (RT-PCR)

cDNA was synthesized using Viva cDNA Synthesis Kit (Vivantis Technology Sdn Bhd, Malaysia) according to the manufacturer's protocol. The mixture contained 10 μg of RNA, 10 mM of dNTP mix, 50 ng random hexamer, and 6.5 μL of nuclease-free water. It was centrifuged briefly, incubated at 65°C for 5 mins and chilled on ice for 2 mins. The incubated mixture was supplemented with 4 μL of 5$\times$ Buffer M-MUL, 100 U M-MULV Reverse Transcriptase, and topped up to 20 μL with Nuclease-free water. It was briefly centrifuged and incubated at 42°C for 60 mins. The reaction was terminated at 85°C, and the cDNA was stored at –20°C.

PCR amplification was performed with the ratio of 2.5 μL of cDNA in 50 μL of PCR mixture that consisted of 2 μL of 10 mM of each specific primer pairs Table 1 for *F. odoratissimum mycobunya-like virus 1*, FoMYV1 (Accession No.: PP934671) and *F. odoratissimum unclassified RNA virus 1*, FoURV1 (Accession No.: PP781703), 25 μL of 2$\times$ Rapid Green Taq Master Mix (Vazyme, Nanjing, China) and the remaining volume was topped with sterile distilled water. PCR was performed using a program consisting of an initial denaturation of 95°C for 3 mins, followed by 40 cycles of 95°C for 10 s, 57°C for 30 s, and 72°C for 30 s, and the final extension at 72°C for 3 mins. The resulting PCR amplicons were analyzed by electrophoresis in 2.5% (w/v) agarose gel containing FloroSafe DNA stain (1st Base Sdn. Bhd, Malaysia) at 60 V/cm for 60 mins.

Pathogenicity test

FO isolates P2S, POH27, and CP2S were cultivated in a 4% (w/v) fresh mung bean solution for 1 week at room temperature before being adjusted to 1×10^6 conidia/ml for inoculation into 8-week-old 'Cavendish' banana (AAA) cultivars.[10] A total of five root tips from each plant were removed. The roots were submerged in the conidial suspension for 30 mins before being transplanted to polybags containing 2 parts of topsoil to 1 part of sand. Each treatment consisted of a negative control (no inoculation) and plants inoculated with respective FO isolates, namely P2S, POH27, and CP2S, with five banana plants for each isolate as biological replicates. The banana plants were watered every day with 100 mL of distilled water, and 2 g of NPK 15:15:15 fertilizer was supplemented once every two weeks. After 6 weeks, the corm discoloration was rated based on established disease severity scoring (0 = No Symptoms; 1 = Isolated point discoloration in vascular tissue; 2 = Discoloration up to 1/3 of the vascular tissue; 3 = Discoloration between 1/3 to 2/3 of the vascular

tissue; 4 = Discoloration greater than 2/3 of the vascular tissue; 5 = Total discoloration of vascular tissue).[25] The test was repeated thrice to ensure reproducibility.

Preparation for enzyme induction media

The enzyme-induction media were prepared with specific substrates using different carbon sources, such as cellulase (Carboxymethyl cellulose (CMC), Nacalai Tesque, Inc., Japan), xylanase (Xylan, Apollo Scientific, UK), and pectinase (Pectin, Sisco Research Laboratories Pvt. Ltd.).[9] The medium contained 1 % (w/v) substrate, 0.1 % (w/v) KH_2PO_4. 0.05 % (w/v) $MgSO_4$, 0.05 % (w/v) KCl, and 0.02 % (w/v) peptone. On the other hand, the media for protease consisted of 1.5 % (w/v) casein hydrolysate, 0.1 % (w/v) K_2HPO_4, 0.1 % (w/v) KH_2PO_4, 2 % (w/v) glucose, and 1% (w/v) peptone. Production of hydrolytic enzymes was induced by inoculating a 5 mm FO mycelial plug from a seven-day-old PDA into a 10 mL broth containing specific substrates. It was incubated on a shaking incubator at 200 rpm at room temperature. The crude enzymes were collected after incubation on days 1, 3, 5, and 7. A volume of 5 mL broth suspension was collected and centrifuged at 12000 × g, 4°C for 5 mins. The supernatant was kept as crude enzymes and stored at –20°C until analysis.

PCWDE assay with 3,5-dinitrosalicylic acid (DNS) colormetric assay

Prior to the assay, the respective substrates, namely 0.5 mm diameter filter paper (WhatmanTM) (cellulase assay), 1% (w/v) CMC (endoglucanase assay), 1% (w/v) microcrystalline cellulose (exoglucanase assay), 1% (w/v) pectin (pectinase assay), and 1% (w/v) xylan (xylanase assay), were dissolved in 0.05 M sodium citrate buffer.[9]

A volume of 300 μL crude enzyme was added with an equal volume of substrates. The mixture was incubated at room temperature for 15 mins. The reaction was stopped with 300 μL of DNS reagent,[9] followed by incubation for 10 mins in a 95°C water bath. All enzymatic assays were repeated thrice. The amount of sugar released was analyzed by measuring the absorbance at 540 nm using a microplate reader (FLUOstar Omega Microplate Reader, Switzerland). Reducing sugars such as glucose (cellulase, exoglucanase and endoglucanase), polygalactorunic acid (pectinase) and xylose (xylanase) were used to plot standard curve for each assays.[9] Only standard curves with $R^2 > 0.98$ will be used to determine enzyme activity (Fig. S1–S3). The enzyme activity was expressed as U/mL/min using the following formula:

$$PCWDEs\ activity = \frac{Absorbance\ of\ Sample}{Gradient\ of\ Standard\ Curve \times\ reaction\ time\ (min) \times\ final\ volume\ of\ sample\ (mL)}$$

Protease assay

The protease activity was determined with azocasein as substrate.[9] Azocaesin solution was prepared with 1% (w/v) azocaesin and dissolved in 0.2 M Tris-HCl (pH 7.4). A volume of 250 μL of crude enzyme was added to 250 μL of 1% (w/v) azocasein solution. The mixture was incubated at room temperature for 30 mins. The reaction was terminated by adding 500 μL of 10% (w/v) trichloroacetic acid (TCA) to the mixture and incubated at room temperature for 10 mins. The mixture was centrifuged at 4000 × g for 2 mins, and 800 μL of supernatant was separated into a new tube. A volume of 250 μL of 1.2 M NaOH was added to the supernatant and mixed thoroughly. All reactions were carried out in triplicate. Absorbance was measured with a Genesys 10S UV-VIS spectrophotometer (Thermo Scientific™, United States) at 440 nm. The protease activity, units of enzymatic activity per mL, was calculated according to the following equation:

$$Protease\ activity\ \left(\frac{U}{mL}\right) = \frac{A \times Vt}{Ve \times 0.01}$$

Where A are the differences of absorbance reading between blank and crude enzymes, Vt is the total reaction volume, and Ve is the volume of crude enzymes.[23]

Statistical analysis

For enzymatic assays, a total of 3 biological replicates were used to extract crude enzymes. A total of 3 spectrophotometric readings (technical replicates) were performed for each biological replicate to control variability. Statistical analysis was carried out using Statistical Analysis Software (SAS v.9.4). Levene's test was conducted to evaluate the homogeneity of variance of the datasets prior to ANOVA analysis (Tables S1–S6). The null hypothesis significance testing was carried out using the Tukey HSD test with $p < 0.05$.

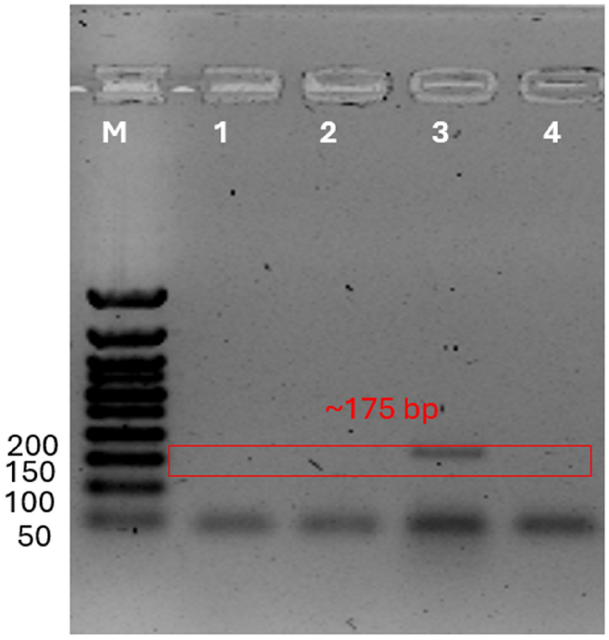

Fig. 1. RT-PCR amplification of mycovirus strain FoMYV1 from P2S isolate producing an amplicon of ~175 bp. Lane M indicates 50 bp ladder; Lane 1 – non-template control; Lane 2 – POH27 (negative control); Lane 3 – P2S (positive control); Lane 4 – CP2S.

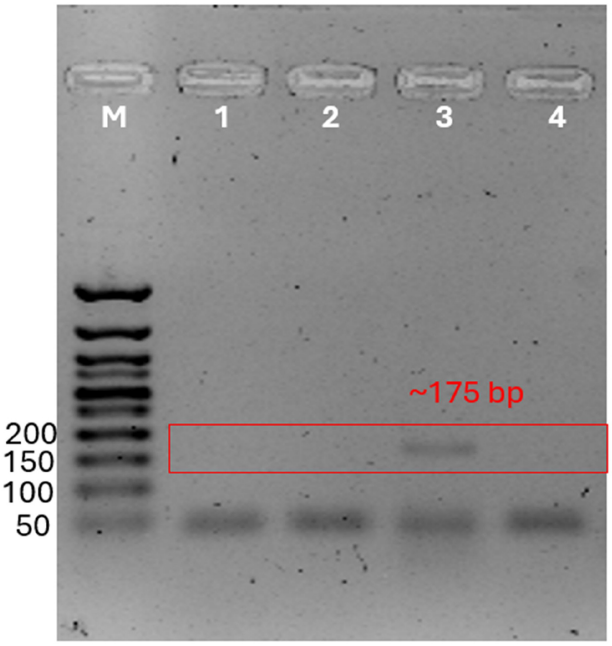

Fig. 2. RT-PCR amplification of mycovirus strain FoURV1 from P2S isolate producing an amplicon of ~175 bp. Lane M indicates 50 bp ladder; Lane 1 – non-template control; Lane 2 – POH27 (negative control); Lane 3 – P2S (positive control); Lane 4 – CP2S.

Results

RT-PCR amplification of mycoviruses

The concentration and purity of the RNA extracted from isolates POH27, P2S, and CP2S were within the acceptable range and ratio (Table S7 and Fig. S4). Subsequently, RT-PCR amplification assay was conducted to detect known and characterized FO mycoviruses, namely FoMYV1 and FoURV1. Only P2S showed amplifications of FoMYV1 (~175 bp) Fig. 1 and FoURV1 (~175 bp) Fig. 2, while POH27 and CP2S (cured P2S isolate) showed no amplification for both mycovirus. A pairwise alignment of sequences derived from PCR amplicons and metagenome datasets confirmed the presence of FoMYV1 and FoURV1 (Fig. S5 and S6).

Disease severity assessment

Based on the severity of corm discoloration Fig. 3, P2S showed reduced discoloration compared to the POH27 isolates. Based on Table 2, the P2S strain co-infected with FoMYV1 and FoURV1 demonstrated lower disease severity (45.0 ± 12.6%) compared to the virulent POH27 strain (72.0 ± 5.8%) and the CP2S strain (90.0 ± 5.8%).

Quantification of cellulase enzymes

Cellulases are classified into multiple enzymes, such as cellulase, endoglucanase, and exoglucanase, which are fundamental in the hydrolysis of cellulose into glucose as a carbon source for fungal growth. Comparison of P2S Fig. 4A, Table S8 to CP2S revealed a similar trend with no significant difference on days 3 and 5, but appeared to be significantly reduced on days 3 and 7. On the other hand, POH27 showed an overall higher cellulase activity compared to P2S and CP2S isolates.

For exoglucanase activity Fig. 4B, Table S9, POH27 indicated a steady increase until day 7. Exoglucanase activity for P2S and CP2S had similar trends, but P2S was significantly reduced when compared to CP2S. On day 3, P2S appeared to have significantly higher exoglucanase activity compared to POH27. However, P2S had an overall decrease in exoglucanase activity on day 5 and day 7, which were significantly lower than POH27. On the other hand, CP2S displayed an overall higher enzymatic activity than P2S and POH27 throughout the 7 days of incubation.

Table 2. Severity of corm discoloration caused by FO isolates P2S, POH27, and CP2S.

Disease Severity	
Treatment	Disease severity percentage (%)
Control	0 [a]
P2S	45.0 ± 12.6 [b]
POH27	90.0 ± 5.8 [c]
CP2S	90.0 ± 5.8 [c]

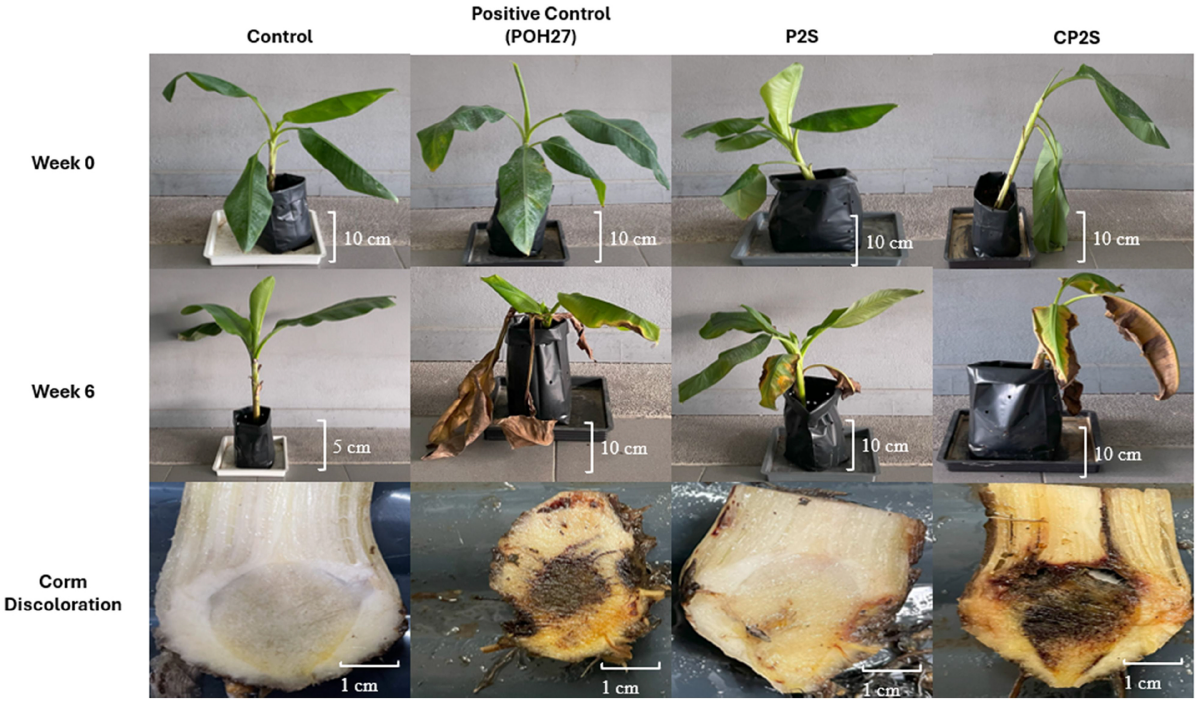

Fig. 3. Pathogenicity test after 6 weeks with 3 treatments consisted of negative control (No FO), FO isolates POH27, P2S, and CP2S. Disease severity was computed based on the corm discoloration score.

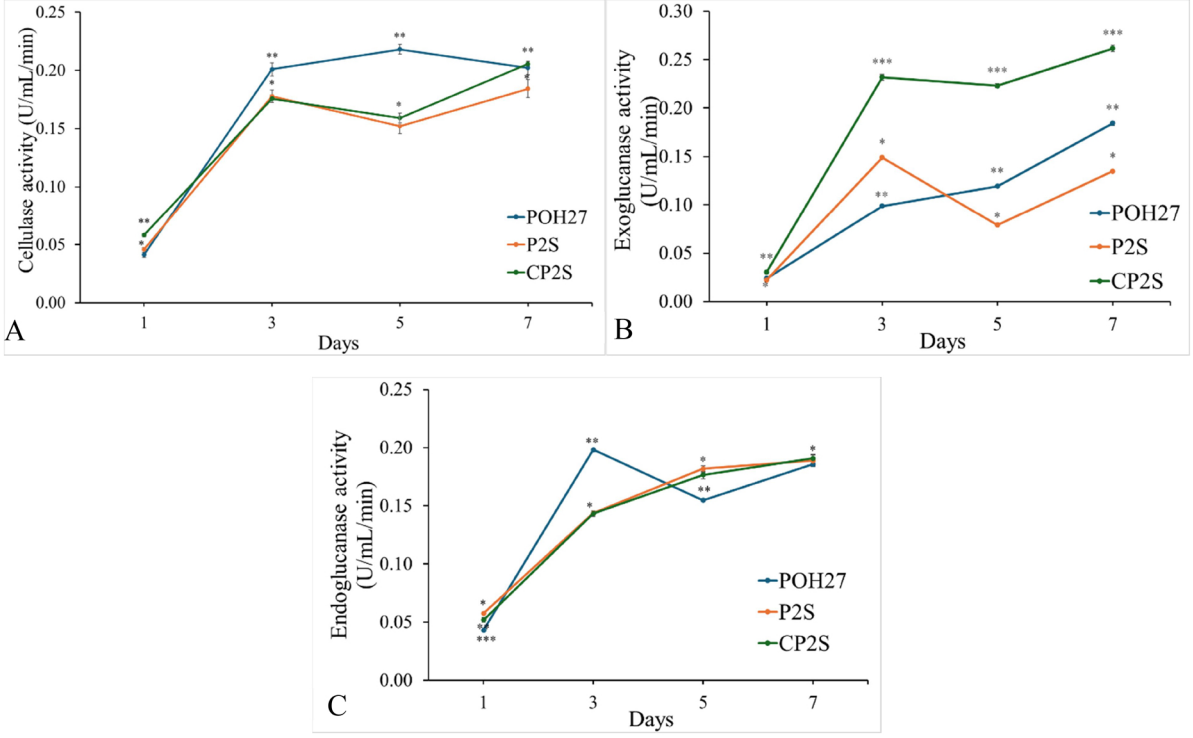

Fig. 4. The enzyme activities of (A) cellulase, (B) exoglucanase, and (C) endoglucanase of P2S, POH27, and CP2S over 7 days of incubation at room temperature. Significant differences of enzymatic activity were indicated by single, double, and triple asterisks (*) between P2S, CP2S, and POH27 isolates at a specific day of incubation using Tukey's test ($p < 0.05$). Each data points represent mean ± standard deviation ($n = 9$)

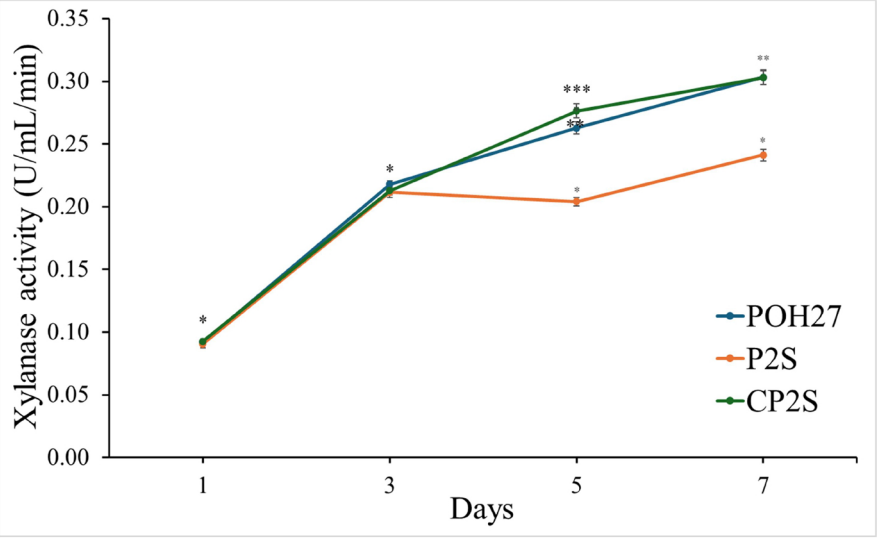

Fig. 5. The enzyme activities of xylanase, of P2S, POH27, and CP2S over 7 days of incubation at room temperature. Significant differences of enzymatic activity were indicated by single, double, and triple asterisks (*) between P2S, CP2S, and POH27 isolates at a specific day of incubation using Tukey's test ($p < 0.05$). Each data point represents the mean ± standard deviation ($n = 9$).

Generally, the endoglucanase activity Fig. 4C, Table S10 recorded a steady increase for P2S, CP2S, and POH27. On day 3, POH27 showed a sharp increase in endoglucanase activity compared to P2S and CP2S. On Day 5, POH27 indicated a steep decrease in endoglucanase activity while P2S and CP2S had a steady increase with no significant difference shown. On day 7, the endoglucanase activity of POH27, CP2S, and P2S isolates was not statistically significant.

Xylanase enzyme quantification

Generally, xylanase activity showed steady increase of enzymatic activity for all three isolates Fig. 5, Table S11. However, P2S had no differences in enzyme activity on days 1 and 3 when compared to CP2S and POH27. On days 5 and 7, P2S enzymatic activity decreased significantly when compared to CP2S and P2S, while CP2S and POH27 increased similarly in xylanase activity on days 5 and 7.

Pectinase enzyme quantification

CP2S and P2S have similar pectinase activity Fig. 6, Table S12 across day 3 to day 7 ($p < 0.05$), whereas POH27 showed significantly higher activity on day 5 to day 7 when compared to CP2S and P2S. On day 3, pectinase activity appeared to have increased insignificantly for all isolates ($p > 0.05$). Pectinase activity was significantly reduced for P2S and CP2S compared to POH27 on days 5 and 7 ($p < 0.05$).

Protease enzyme quantification

Protease activity Fig. 7, Table S13 had a similar trend among all isolates. Protease activity was higher on day 1 compared to day 3 since all isolates showed a decrease in protease activity. While on days 5 and 7, all isolates' protease activity increased. In general, the protease activity of CP2S was significantly higher than that of P2S and POH27 isolates from day 1 to day 7. By comparing P2S and POH27 isolates, the protease activity of P2S was significantly lower on days 5 and 7.

Discussion

In this study, coinfection of FoMYV1 and FoURV1 in the P2S isolate reduced the disease severity of corm discoloration and PCWDE activity. Mycovirus infection in P2S was potentially linked to the reduction of enzyme activity, such as cellulase, exoglucanase, xylanase, and protease. Non-mycovirus-infected strains such as POH27 and CP2S did not show a significant difference in the overall enzymatic activity, which may be due to the origin of the isolates. FO isolated from different geographical regions might have a different enzymatic activity profile. For instance, CHV1 infecting *C. parasitica* with different regional isolates has a different enzymatic profile because CHV1 could only exert its hypovirulent effect on certain regional isolates of C. parasitica, whereas other isolates remain asymptomatic.[26] The co-infection of FoMYV1

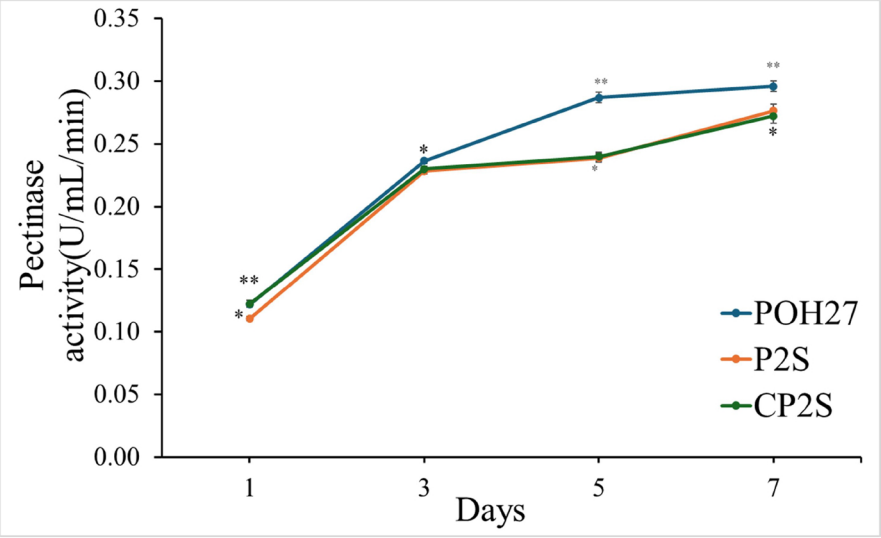

Fig. 6. The enzyme activities of pectinases of P2S, POH27, and CP2S over 7 days of incubation at room temperature. Significant differences of enzymatic activity were indicated by single, double, and triple asterisks (*) between P2S, CP2S, and POH27 isolates at a specific day of incubation using Tukey's test ($p < 0.05$). Each data points represent mean ± standard deviation ($n = 9$).

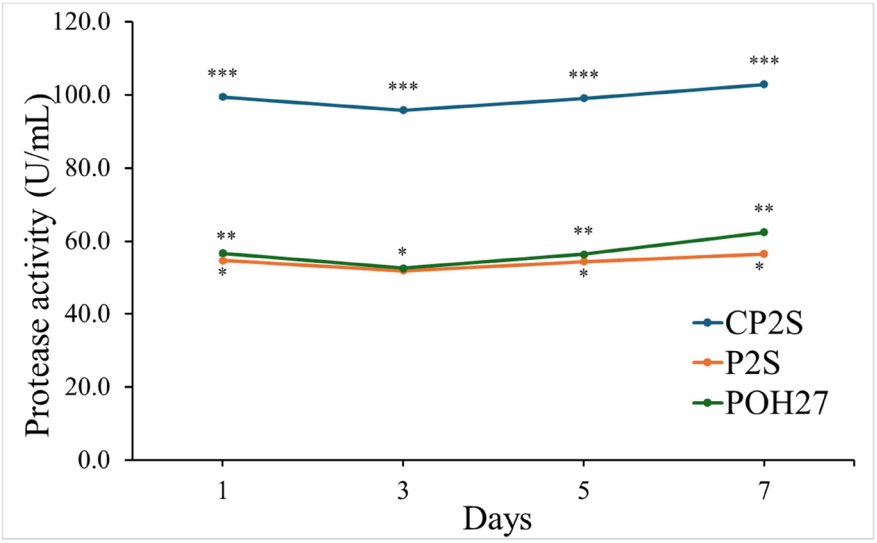

Fig. 7. The enzyme activities of proteases of P2S, POH27, and CP2S over 7 days of incubation at room temperature. Significant differences of enzymatic activity were indicated by single, double, and triple asterisks (*) between P2S, CP2S, and POH27 isolates at a specific day of incubation using Tukey's test ($p < 0.05$). Each data point represents the mean ± standard deviation ($n = 9$).

and FoURV1 of P2S is likely to have contributed to the reduction of PCWDE activity, which could have affected host penetration. FO requires PCWDEs for successful penetration and subsequently, colonization of its host.[27] Other fungal pathogens, such as S. sclerotium infected by the hypovirulent SsHADV-1, exhibited downregulated PCWDEs expression levels, contributing to the reduced pathogenicity towards the plant host.[16] Furthermore, metabolic profiling of *C. parasitica* infected by CHV1 mycovirus revealed low laccase activity, an important enzyme in lignin breakdown of chestnut trees, resulting in the reduction of the pathogen's virulence.[14,26]

Cellulases such as exoglucanase contributed to the degradation of plant cell walls during the penetration of the pathogenic fungus by cleaving a smaller chain of cellulose that was initially broken down by endoglucanase.[28] FoMYV1 and FoURV1 co-infection could have reduced exoglucanase production, which contributed to the decrease of cellulase activity, leading to poor host penetration. It is also worth noting that endoglucanase activity of the virulent

POH27 isolate spiked from day 1 to 3 but dropped drastically on day 5. The biphasic enzyme activity could be due to the rapid accumulation of soluble sugar or cellobiose, a byproduct of endoglucanase activity, resulting in enzyme repression on day 5.[29] As the byproducts were metabolized by exoglucanase, the endoglucanase activity increased again on day 7. In contrast, the P2S isolate had higher exoglucanase activity compared to POH27 on days 1 and 3, but the enzyme activity plunged on day 5, and subsequently, the activity remained low on day 7. The fluctuating enzymatic pattern of P2S suggests the hypovirulence exerted by the co-infection of mycovirus.

Xylanase hydrolyzes xylan, a fundamental component for cell wall and vascular tissue development.[30] Studies revealed that xylanase produced by pathogenic fungi such as *Botrytis cinerea* and *Verticillium dahliae* is an important pathogenic effector in causing cell death on the host plant.[30] Xylanase might have contributed to FO virulence activity by triggering cell death in the host plant, as observed in higher enzymatic activity in isolates POH27 and CP2S than P2S. Nonetheless, an in-depth study is necessary to characterize the functional role of this enzyme in the pathogenicity of FO.

Proteases are known as one of the many pathogenicity factors in FO. It is suggested that FO's protease family, such as metalloprotease 35, might play an important role in causing the pathogenicity of FO towards banana plantlets. The deletion of this pathogenicity gene suppressed the virulence activity of FO. Since metalloprotease 35 was known to degrade chitinase produced by plants in response to fungal invasion, deletion of this gene was correlated with the improved host tolerance towards FO.[31–33] Therefore, reduced protease activity in P2S might have contributed to the ineffectiveness of breaking down the plant's chitinase, an important defense enzyme against FO. Thus, FoMYV1 and FoURV1 co-infection is suggested to exert a hypovirulence effect towards FO. However, it is not known whether a single or both mycovirus strains were required to induce hypovirulence. The effect of single and co-infection of FoMYV1 and FoURV1 on the P2S isolate on the production of PCWDE, coupled with transcriptomics or proteomics datasets, should be studied in the future to further elucidate the mechanisms of mycoviral hypovirulence on the host gene regulation.[15,17,21,34]

Conclusion

The co-infection of FO isolate P2S with FoMYV1 and FoURV1 reduced disease severity significantly and likely inhibited the activities of certain key plant cell wall-degrading enzymes, including exoglucanase, xylanase, and protease. The reduced enzyme activity in the P2S isolate could have impaired the invasion and colonization of host tissues, thereby resulting in the hypovirulent phenotype. Future studies should investigate the effects of single viral infection of FoMYV1 and FoURV1 to pinpoint their roles in PCWDE suppression. These findings provided new insights into the possible role of mycoviruses in modulating fungal pathogenicity and suggested their promising potential as biocontrol agents for the management of Fusarium wilt of banana.

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Authors' declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images that are not ours have been included with the necessary permission for republication, which is attached to the manuscript.
- No animal studies are present in the manuscript.
- No human studies are present in the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee at Universiti Tunku Abdul Rahman, Malaysia.

Authors' contributions statement

Z.Q.L. conducted the experiment, collected and analyzed the data, wrote the draft, and revised the manuscript.; C.K.F.W.; N.I.B.M.I.; G.V., supervised all the previous steps and proofread the manuscript.

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Supplementary materials

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تأثير الإصابة بالفيروسات الفطرية على نشاط الإنزيمات التحلّلية في فطر Fusarium oxysporum f. sp. cubense السلالة المدارية 4، المُسبِّب لذبول الفيوزاريوم في الموز

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الملخص

يُعَدّ فطر Fusarium oxysporum f. sp. cubense السلالة المدارية 4، المعروف أيضاً باسم Fusarium odoratissimum (FO)، من المُمرضات الفطرية التي تُصيب أصناف الموز (Musa spp.). ويقوم هذا الفطر بإفراز إنزيمات محلِّلة لجدار الخلية النباتية (PCWDEs) لتمكينه من اختراق الجذور واستعمارها، مما يؤدي إلى ظهور أعراض الذبول وموت العائل. الفيروسات الفطرية (Mycoviruses) هي فيروسات تُصيب الفطريات، وقد تبيّن أن بعضها يمكن أن يعمل كعامل مكافحة حيوية عبر إحداث ضعف الضراوة (Hypovirulence) في المُمرضات الفطرية، وهو عرض مُميّز لانخفاض قدرات الإمراض في العائل المصاب. كما ارتبط ضعف الضراوة في عدة مسببات فطرية نباتية بانخفاض نشاط إنزيمات PCWDE. حتى الآن، لم يُبلَّغ عن وجود فيروس فطري مُضعِف للضراوة في FO. لذلك، أُجريَت هذه الدراسة لتمييز سلالة محددة من FO وهي العزلة P2S المصابة بشكلٍ مزدوج بكلٍّ من فيروس FO الشبيه بفيروسات Mycobunyavirales (FoMYV1) و فيروس FO غير المصنّف من نوع RNA (FoURV1)، وذلك من حيث ضراوتها وإنتاجها لإنزيمات PCWDE، مقارنةً بالعزلتين غير المصابتين وعاليتي الضراوة POH27 و CP2S. أظهرت النتائج أن FoMYV1 وFoURV1 خفّضا شدة المرض إلى 45.0 ± 12.6% في عزلة P2S مقارنةً بعزلتي POH27 (90.0 ± 5.8%) وCP2S (90.0 ± 5.8%). علاوة على ذلك، يُحتَمَل أن الإصابة المزدوجة بالفيروسات الفطرية وانخفاض شدة المرض يرتبطان بتثبيط إنتاج إنزيمات PCWDE مثل الإكسوغلوكاناز، والزيلاناز، والبروتياز. تُعَدّ هذه الدراسة الأولى التي تقترح آلية محتملة لضعف الضراوة ناجمة عن الإصابة المزدوجة بالفيروسات الفطرية في FO.

الكلمات المفتاحية: FoMYV1، FoURV1، ضعف الضراوة (Hypovirulent)، الفيروسات الفطرية (Mycovirus)، إنزيمات تحلُّل الجدار الخلوي النباتي.