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Bacterial biocontrol against Fusarium wilt in Pisang Awak (Namwa) Banana

Palisa Mahachai[1], Orapan Meesungnoen[1], Wandee Wattanachaiyingcharoen[2] and Pakpoom Subsoontorn[1,*]

[1]Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand
[2]Faculty of Science, Naresuan University, Phitsanulok, Thailand
[*]Corresponding author: pakpoomton@gmail.com

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Abstract

Pisang Awak is one of the most important banana cultivars due to its drought tolerance and nutritional value. Nonetheless, Pisang Awak is highly susceptible to Fusarium wilt caused by *Fusarium oxysporum* f.sp. cubense (*Foc*). Over the past decades, the use of biocontrol bacteria against *Foc* has shown promising results in both greenhouse and field trials. However, none of these previous studies demonstrated effective control of Fusarium wilt in Pisang Awak. We show that *Pseudomonas* and *Bacillus* strains isolated in Thailand can suppress *Foc* mycelial growth *in vitro* and significantly mitigate the severity of Fusarium wilt in Pisang Awak (Namwa) banana in a greenhouse experiment. With these bacterial treatments, Fusarium wilt incidences can be reduced by up to 70% and 89% in young and old banana plants, respectively. We have also reported the first bacterial community analysis of Pisang Awak rhizospheres in response to *Foc* and bacterial biocontrol. The abundance of several bacterial families such as *Rhodospirillaceae*, *Bradyrhizobiaceae* and *Hyphomicrobiaceae* increased in rhizospheres inoculated with *Foc* and decreased in rhizospheres treated with biocontrol. These families were previously shown to be associated with *Foc* suppressive soil. These results provide the basis for the development of bacterial biocontrol products against *Foc* in Pisang Awak banana.

Keywords: Microbiome, Rhizosphere, *Bacillus*, *Pseudomonas*, *Foc*

1. Introduction

Bananas are the most consumed fruit crop in the world, providing a stable source of calories and income for millions of people, particularly in developing countries [1]. However, banana production worldwide has been threatened by pathogenic fungi named *Fusarium oxysporum* f.sp. cubense *(Foc)* [2]. Once a plantation is completely infested, 100 percent yield loss is possible if not properly controlled. *Foc* spores can persist in the soil for up to 30 years and can spread for a great distance via planting materials or contaminated soil [3].

Over the past four decades, nearly half of published studies on *Foc* management proposed the use of biological controls [4]. Most of these studies used microbial agents, particularly *Bacillus*, *Pseudomonas*, and *Trichoderma*. These microbial agents reduce incidences and severity of *Foc* infection via a variety of mechanisms that range from direct antibiosis to competition for nutrients and induction of the plant defense mechanisms [5]. Most biocontrol experiments were conducted in only a few banana cultivars. For example, the uses of *Pseudomonas* have been demonstrated almost exclusively in Rastali banana (*Musa* spp. AAB) in India. Most *Bacillus* biocontrol was demonstrated only in Gros Michel or Cavendish (*Musa* spp. AAA). The banana cultivar is a key determinant of Fusarium wilt severity and is likely to have significant impacts on the effectiveness of biocontrol systems.

Pisang Awak or Namwa cultivar (*Musa* spp. ABB) is known for its drought tolerance and nutritional content, making it an important food crop in arid areas with poor soil quality such as Africa and Southeast Asia [6]. Thailand has over 64,000 hectares of Namwa plantations, accounting for over three forth of all banana plantations [7]. Unfortunately, Pisang Awak is highly sensitive to *Foc* infection. In comparison to other popular cultivars such as Cavendish, Pisang Awak xylems are invaded by *Foc* more quickly, resulting in more severe wilting rates [8]. Still, there is currently no published study on the development and evaluation of *Foc* biocontrol agents for this cultivar.

Here, we reported an evaluation of *Pseudomonas* and *Bacillus* strains that are capable of inhibiting *Foc* in Pisang Awak (Namwa) bananas. Both strains can suppress *Foc* mycelial growth *in vitro* and reduce the severity of *Fusarium* wilt in Namwa banana plants in a greenhouse experiment. Since previous reports have shown the correlation between *Foc* severity and certain microbial taxa in the banana rhizosphere [9-11], we also analyzed bacterial compositions in rhizospheres of Namwa without *Foc* spore inoculants, with *Foc*, and with these bacterial biocontrol agents. We showed that *Foc* and bacterial biocontrol agents affected the overall diversity and the abundance of several rhizosphere bacteria previously known to be associated with *Foc* suppressive soil.

2. Materials and methods

2.1 Biocontrol strains of Pseudomonas and Bacillus

Commercial biocontrol *Bacillus* strains (BionBac™ and BsBomb™) were purchased from manufacturers the ICP Ladda Co. Ltd. and Kasetkawna, Thailand. *Pseudomonas fluorescens* TISTR 1887 (PfT87) and *P. fluorescens* DSM50090 (PfD90) were purchased from Thailand Institute of Scientific and Technological Research and Leibniz Institute DSMZ, Germany, respectively. *Bacillus siamensis* Bs36 (Bs36) was isolated from the roots of Namwa banana grown at Plant Propagation Center 6th, Phitsanulok, Thailand, and identified using the 16S rRNA gene (Macrogen, Inc. Korea). The isolation protocol was modified from Vlassak et al. (1992) [12].

2.2 In vitro Foc biocontrol test

Five *Bacillus* or *Pseudomonas* (Table 1) were tested for their antagonistic effects against *Foc* race 1 strain A1736 (from Department of Agriculture, Thailand) by dual culture assay [13]. In brief, 5 mm discs were cut from the edge of 7 days old *Foc* that were grown on potato dextrose agar (PDA) culture and were placed at the center of a new PDA plate. The bacteria culture that grew overnight in the Nutrient Broth (NB) medium was streaked at a distance of 2 cm from the center of the *Foc* disc. The plates were incubated at room temperature (25-30 ℃) and the inhibition zones were measured after 7 days. The percent inhibition (PI) was calculated using a formula given by Vincent (1947): PI = C-T/C × 100, where PI = percent inhibition, C = Radius of *Foc* colony in absence of antagonist (cm), and T = Radius of *Foc* colony in the presence of antagonist bacteria (cm) [14].

2.3 Preparing Foc spore and bacterial biocontrol agents

To prepare *Foc* conidia suspension, the fungus was grown on the PDA for 14 days in a dark at room temperature (25-30 ℃). The fungal culture was spread in 10 mL of 0.1% tween 80 to collect the spore. The spores were stored at 4 ℃ and re-suspended in sterile water to a concentration of 10^5 spores/mL before use. To prepare bacterial biocontrol agents, bacteria stocks were grown in NB medium at 37 ℃ in a 200-rpm shaker (except for PfD90, where the temperature was 30 ℃). After 16 hours of incubation, the bacterial cultures were spun down at 5000 rpm for 15 minutes. The supernatant was discarded, and the cell pellet was re-suspended in distilled water, and the concentration was adjusted as 10^8 CFU/mL [15].

2.4 In vivo Foc biocontrol test in a greenhouse

Biocontrol agents were tested against *Foc* in Pisang Awak (Namwa) banana plant. An grown in a greenhouse at Naresuan University, Thailand, from March 2019 to February 2020. A ambient temperature in the greenhouse, was between 34 ℃ to 44 ℃ with 30-70% relative humidity. Banana plantlets from tissue cultures were purchased from Plant Propagation Center, Suphan Buri, Thailand. These plantlets were rested for 7 days in the greenhouse before starting the experiment.

The treatments were arranged in a randomized design. The experiment was done in duplicate with 5 banana plants per replicate per treatment. A total of five treatments were used for the study: 1) no treatment, 2) *Foc* alone, 3) *Foc* + Bs36, 4) *Foc* + PfD90, and 5) *Foc* + PfT87. These experiments were performed on young (8-10-week-old) and old (12-17-week-old) banana plants. *Foc* biocontrol experiment procedure was adapted from [15]. Briefly, each banana plant was gently lifted off the soil and washed with running tap water. A 1 mm tip of the largest root of each plant was excised to facilitate *Foc* invasion. For biocontrol treatments (treatment #3 - #5), banana roots were dipped into 10^8 Colony Forming Unit (CFU)/mL bacterial suspension (DSM50090, TISTR 1887, or Bs36) for 1 hour and then dipped into 10^5 spores/mL. *Foc* conidial suspension was maintained for another 1 hour before replanting into 3.5 kg non-sterilized organic soil (purchased from Sor Moonsub, Phitsanulok, Thailand) in polythene bags. For no *Foc* control (treatment #1), banana roots were dipped into sterile water instead of conidia or bacterial suspension. For no biocontrol treatment (treatment #2), banana roots were dipped into conidia without any prior exposure to bacterial antagonists. After two weeks, additional 200 mL of 10^8 CFU/mL bacteria suspension was poured onto the soil of each biocontrol treatment pot.

At the end of 4 weeks, the incidence and severity of Fusarium wilt were measured. Each banana plant was cross-sectioned at the ground level. The severity of Fusarium wilt was reported as the percentage of rhizome cross-section area that discolored (i.e., turning from white to yellow, brown, or black). Incidences of Fusarium wilt were reported as the percentage of banana plants per replicate that had any observable Fusarium wilt symptom. Statistical analysis was conducted including that done by Turkey of the difference in Fusarium wilt severity across different treatments [16,17].

2.5 Analysis of bacterial community in the rhizosphere

Three young banana pots were chosen randomly from each treatment group at the end of the 4-week experiment. Rhizosphere from the near soil surface down to 30 cm depth was taken for metagenome DNA extraction. Next, the DNA was extracted from each 250 mg soil sample according to DNeasy PowerSoil Pro Kit (Biodesign Co., LTD.). 16s rRNA gene amplicon metagenomic sequencing was conducted by Macrogen (South Korea) on the V3-V4 region with at least 50,000 data reads per sample. Demultiplexed sequencing results were analyzed and visualized using the QIIME2 microbiome bioinformatics pipeline [18]. Taxonomic assignment was performed using pre-trained GreenGene classifier (gg-13-8-99-nb-classifier) [19]. Heatmaps were generated using R Statistical Computing software [17].

3. Results

We isolated bacterial clones from rhizoplanes and the endorhizosphere of the Namwa banana. One of these clones with high *Foc* inhibition activity, named *B. siamensis* 36 (Bs36), was chosen for further study *in vitro* and in plants. We also included two potential *Foc* biocontrol *P. fluorescens* in our study. *P. fluorescens* TISTR 1887 (PfT87) was previously isolated from rhizomes of cauliflower in Thailand and have biocontrol activity against pathogenic fungi in rice, cassava, and corn [20-22]. *P. fluorescens* DSM50090 (PfD90) is a type of strain under *P. fluorescens* with the full genome sequence. While no previous work reported biocontrol activity of this strain against pathogenic fungi, it has been shown to promote the growth and seed yield of maize [23,24].

We performed dual culture inhibition assay between antagonistic bacteria and Foc race 1 strain A1736 on PDA. PfT87 had the highest average *Foc* mycelium growth inhibition at 50.5%, followed by Bs36 having an average of 22.5% (Table 1). *P. fluorescens* DSM50090 cannot inhibit *Foc* mycelium growth on PDA. Two *Bacillus subtilis* strains from commercial biocontrol products, BsBomb™ and BionBac™, had similar or lower Foc growth inhibition activity than Bs36. PfT87 had higher *Foc* growth inhibition than both commercial strains ($p < 0.001$).

Table 1 *In vitro* antagonism against *Fusarium oxysporum* f. sp. cubense race 1 *(Foc)* on potato dextrose agar (PDA) by selected *Bacillus* or *Pseudomona*s.

Biocontrol agents	Sources	% Foc inhibition*
B. subtilis (BionBac™)	ICP Ladda., Thailand	$8.5^{b} \pm 2.9$
B. subtilis (BsBomb™)	Kasetkawna, Thailand	$20.8^{c} \pm 4.6$
B. siamensis 36	Namwa banana rhizoplane	$22.5^{c} \pm 4.5$
P. fluorescens DSM50090	DSMZ, Germany	$0.0^{a} \pm 0.0$
P. fluorescens TISTR 1887	TISTR, Thailand	$50.5^{d} \pm 0.9$

*These are averaged from triplicate experiments. Different superscripts indicate significant differences ($p \leq 0.05$)

We tested whether Bs36, PfD90, and PfT87 can reduce the incidence and severity of Fusarium wilt in Namwa banana plants. Experiments were conducted on young (8-10-week-old) and old (12-17-week-old) banana plants in a greenhouse. We divided banana plants into these age ranges due to their significant difference in size at the beginning of the experiment (height 6±1.5 cm and 16±1.5 cm, respectively, for young and old banana plants). In control experiments without a biocontrol agent, all young plants and 90% of old plants showed Fusarium wilt symptoms within four weeks after *Foc* inoculation (Figure 1). Average *Foc* severity damage was 54% and 50% in young and old banana plants, respectively. Bs36 treatment reduced the incidence of Fusarium wilt in young and old banana to plants 60% and 10%, respectively; PfD90 treatment reduced the incidence of Fusarium wilt in young and old banana plants to 70% and 40% respectively; PfT87 treatment significantly reduced Fusarium wilt in only young banana plants to 30%. Bs36 and PfD90 significantly reduced the *Foc* severity in only old banana plants to below 5% damage while PfT87 only significantly reduced the *Foc* severity of young banana plants to 9%.

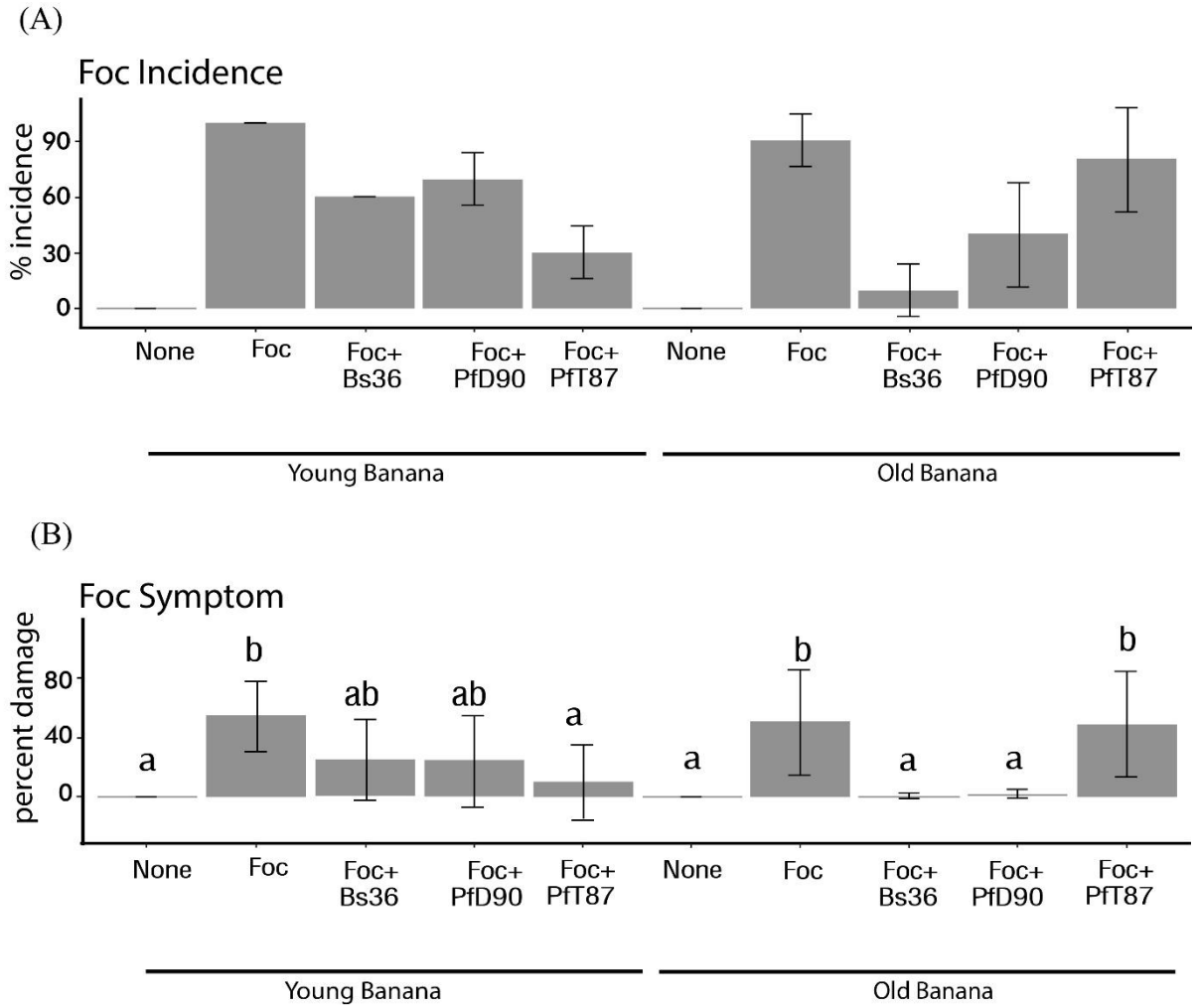

Figure 1 Effects of bacterization with biocontrol *Bacillus* or *Pseudomonas* on Namwa (Pisang Awak) banana plants in a greenhouse, (A) the percentage of Fusarium wilt incidence and the second illustrates the damage percentage on banana plants in each treatment group, (B) percent damage of each banana plant was measured as the percentage of discolored banana rhizome cross-section area. Different letters (a, b, c, …) above each bar indicate statistically significant differences ($p < 0.05$). Bs36 = *B. siamensis* 36; PfT87 = *P. fluorescens* TISTR 1887, and PfD90 = *P. fluorescens* DSM 50090.

Using 16s rRNA amplicon metagenomics, we explored how *Foc* infection and biocontrol treatments affected the bacterial community in rhizospheres of young Namwa banana plants. The rarefaction curves confirmed that all treatment samples had similar operational taxonomic unit (OTUs), and our sequencing counts were sufficient for capturing OTU richness in all our samples (Figure 2A). Overall, alpha diversities were not significantly different across five treatment samples with one exception. Alpha diversity, measured as Shannon's entropy, was significantly lower in *Foc* + Bs36 treatment group than in the *Foc* control group (p-value < 0.05) (Figure 2B). Principle Coordinate Analysis (PCoA) plot showed that bacteria populations in *Foc* + Bs36, *Foc* + PfT87, and *Foc* alone treatment clearly differed from one another (Figure 2C).

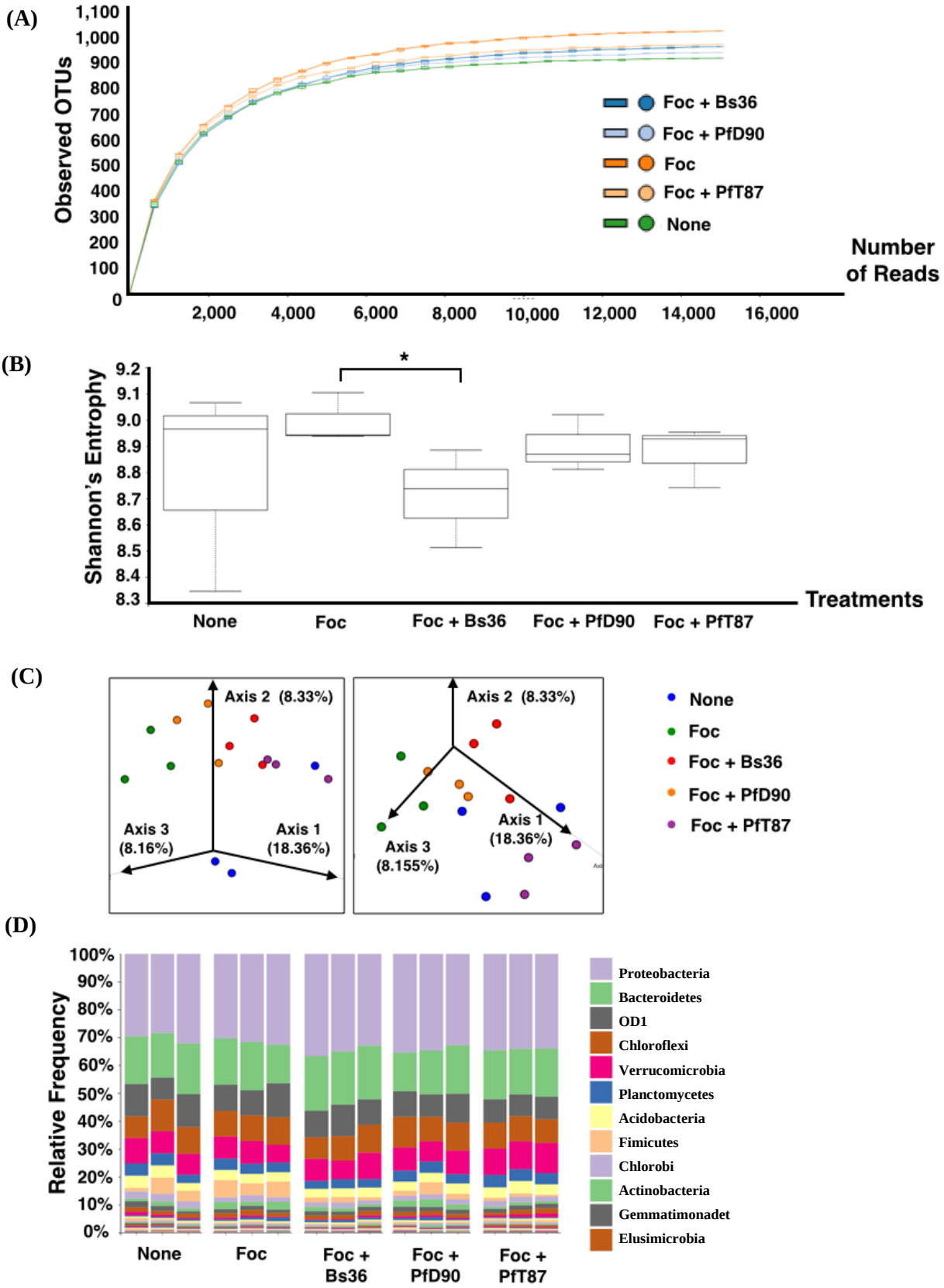

Figure 2 shows the Effects of *Foc* infection and bacterization with biocontrol *Bacillus* or *Pseudomonas* on bacterial rhizospheres of Namwa (Pisang Awak) banana plants. 15 rhizosphere samples (3 rhizosphere samples for each of the 5 treatments) were used for 16S rRNA gene metagenomic sequencing. (A) rarefaction curves, (B) Shannon's alpha diversity, (C) The Principal Coordinates Analysis, (D) abundances of phyla.

Phylogenetic assignment showed that bacterial communities in all treatments were dominated by *Proteobacteria* and *Bacteriodetes* phylum, followed by *OD1, Chloroflexi*, and *Verrucomicrobia* (Figure 2D). There is no apparent difference in the types and fractions of these major phyla across five treatments. Further microbial community analysis at the family level revealed core bacterial families that were present in all rhizosphere samples from all treatments (Figure 3A). The most abundant core family was *Cytophagaceae*, followed by *Opitutaceae, Hyphomicrobiaceae*, and *Chitinophagaceae*. Certain families were only present in samples from some treatments but not others (Figure 3B). For example, *Streptomycetaceae* was absent from all samples in *Foc* + Bs36 treatment but present in all other samples. *Planococcaceae* was absent from all samples in *Foc* + PfT1887 treatment but present in all samples from *Foc* control treatment. We then compared family abundances among different pairs of treatments (Figure 3C). The abundances of several families significantly increased (e.g., *Planococcaceae*, *Bradyrhizobiaceae*, and *Hyphomicrobiaceae*) or decreased (e.g., *Cellulomonadaceae*) in the rhizosphere inoculated with *Foc* relative to the control rhizosphere. Bacterial biocontrol treatments appeared to decrease the abundance of several families (e.g., *Planococcaceae, Thermoactinomycetaceae,* and *Lachnospiraceae*) relative to *Foc* control treatments.

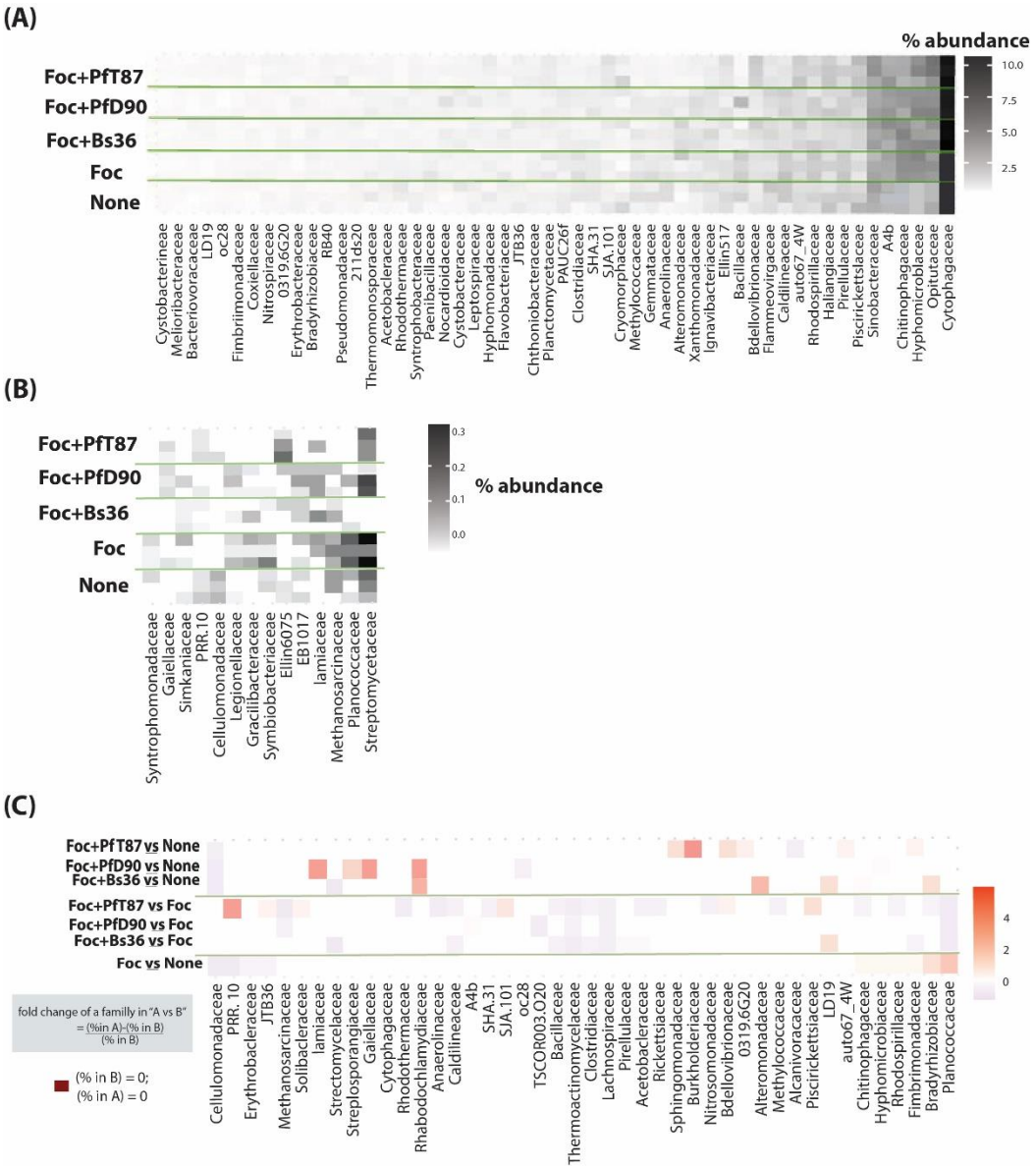

Figure 3 Analysis of family-level abundance. (A) percent abundances of bacterial families present in all samples, (B) percent abundance of bacterial families which were absent from all samples from some treatments, (C) fold changes in family abundance comparing between different pairs of treatments. The white color indicates that the change was not statistically significant. Red and blue color indicate whether the mean abundance of the family significantly ($p < 0.05$) increased or decreased, respectively. Unidentifiable families are not shown.

4. Discussion

The biocontrol agents can reduce Fusarium wilt incidence in young banana plants from 100% to 30% (using PfT87) and in the old banana plant from 90% to 10% (using Bs36). The average Fusarium wilt damage can also be reduced from above 50% to below 10% in both young and old banana plants (using PfT87 and Bs36, respectively). A recent systematic review reported median banana *Foc* biocontrol efficacies of *Pseudomonas* and *Bacillus* at approximately 70% in greenhouses and field trials [4]. Thus, the efficacy of our biocontrol microbes in Namwa is at least on par with that of previous studies done in other banana cultivars. Nonetheless, given that our experiments were only conducted in a greenhouse over relatively a short period (4 weeks), further large-scale studies are required before these microbes can practically be used in the Namwa banana plantation.

The performances of biocontrol agents from *in vitro* dual culture assay were not well-correlated with the performances in banana plants. PfD90 did not inhibit *Foc* mycelium growth on PDA but can significantly reduce Fusarium wilt severity in young banana plants. PfT87 inhibited *Foc* mycelium growth on PDA better than Bs36 did. However, their abilities to reduce Fusarium wilt incidence and severity of Bs36 were higher than PfT87 in old banana plants. Such discrepancy between *in vitro* and plant biocontrol efficacies could be explained by the fact that many factors other than direct antibiosis determine biocontrol efficacy in plants. These factors include root colonization, interactions with other rhizobacteria, indirect biocontrol effects, etc. [5]. For example, many *Pseudomonas* strains including PfD90 are known as plant growth-promoting bacteria and capable of inducing banana plant defense system including expression of peroxidase, polyphenol oxidase, and phenylalanine ammonia-lyase [25,26]. Conventional biocontrol microbe screening often discarded microbes that had weak or no *in vitro* antagonistic effect against targeted pathogens. This workflow is likely to make us miss an opportunity to discover and exploit a vast majority of beneficial microbes that can prevent plant diseases. Alternative screening workflows, for instance, the uses of direct molecular screening for genetic toolset related to biocontrol properties, may help us get around this problem

Only one previous work explored the relationship between bacterial compositions of Pisang Awak rhizosphere and suppression of *Foc* by ground cover [27]. To the best of our knowledge, we are the first to show how the direct inoculation of *Foc* and bacterial biocontrol treatments may affect the bacterial community in the Pisang Awak rhizosphere. *Cytophagaceae* was by far the most abundant family in all our samples. Many of these family members are proficient at the digestion of insoluble cellulose and are often enriched with organic matter treatment [28]. This observation is not surprising given that we used organic fertilizer in the soil in all experiments. Other high abundance core families such as *Hyphomicrobiaceae* and *Sinobacteraceae* were previously reported as top colonizers in the endosphere of banana plants whether or not they were infected by *Foc* [29].

Hyphomicrobiaceae, *Bradyrhizobiaceae*, *Rhodospirillaceae*, *Bacillaceae*, and *Streptomycetaceae* were previously reported to be among the core microbiome of *Fusarium* suppressive soil [9,30]. Interestingly, we found that these families either became more abundant in the rhizosphere inoculated with *Foc* compared to non-inoculated rhizosphere or/and became less abundant in *Foc* + biocontrol rhizosphere compared to the rhizosphere inoculated with *Foc*. It could be possible that some of these family members have an indirect parasitic relationship with *Foc*. Thus, the growth of the bacteria population from these families could benefit from the presence of *Foc* (or other related fungi). In the presence of other potent *Foc* biocontrol agents (such as Bs36 or PfT87), less *Foc* is available causing the reduction of these families' abundance. We observed a similar trend in *Chitinophagaceae*, though this family was reported to be less abundant in *Fusarium* suppressive soil than non-suppressive soil. Some members of this family are endosymbionts of *Fusarium* [31]. Thus, the abundance of the family should be correlated to the abundance of *Foc* as we observed in our experiment

5. Conclusion

We characterized two *Pseudomonas* and one *Bacillus* with biocontrol activities against *Foc* in Namwa (Pisang Awak) banana. These microbes can significantly reduce the incidence of Fusarium wilt in young Namwa banana plants. Biocontrol agents based on *B. siamensis* can also significantly reduce the incidence and severity of Fusarium in old Namwa banana plants and cause the reduction of bacterial alpha diversity compared to controlled Namwa banana inoculated with *Foc* alone. We also found that *Foc* infection and these biocontrol bacteria significantly altered the abundance of several banana rhizobacteria, some of which were previously reported to be associated with *Foc* suppressive soil. Future development of microbial formulation and understanding of the relationship between banana cultivar, rhizobacteria, and biocontrol agents could help us prevent the catastrophic impact of *Foc* outbreaks in Pisang Awak banana plantations.

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