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Morganella sp. : A Dual-Action Biocontrol Agent and Growth Promoter against *Fusarium oxysporum* in Banana

Joni Rey H. Campilan[1,2,*] and Dorothy A. Antesa[1]

[1]Notre Dame of Marbel University, Alunan Avenue, Koronadal City, Philippines 9506
[2]Plant Biology Graduate Program, University of Massachusetts, Amherst, Massachusetts, USA 01003

[*]Corresponding author: jcampilan@umass.edu

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Abstract

Banana Fusarium wilt caused by *Fusarium oxysporum f. sp. cubense* (Foc) remains the most critical banana disease that threatens the industry worldwide. Identifying potential biocontrol agents that support host growth is essential in finding effective solutions against Foc. In this study, bacterial isolates from banana pseudostems, rhizomes, and rhizosphere soil were tested for their antifungal activity against Foc and plant growth-promoting effects under nursery conditions. Visual agar plate assay showed that isolates RS13, ER03, and ER20 significantly inhibited Foc growth on days 5, 6, 7, and 8 compared to *E. coli* ($p<0.05$). Additionally, these isolates promoted overall banana plantlet growth by increasing recorded values for pseudostem diameter, height, leaf number, and total leaf area. To further investigate the mechanisms that underlie its antifungal and plant growth-promoting activities, the whole genome of RS13 was sequenced and analyzed. Genome assembly identified RS13 as *Morganella sp.*, with functional annotations resembling *M. morganii subsp. morganii KT* in functional and metabolic profiles. Gene mining revealed RS13 has increased protein members in the Type III secretion system and three chitinases, indicating its potential biocontrol mechanisms against pathogenic fungi compared to *M. morganii subsp. morganii KT*. Moreover, metabolic predictions indicate RS13's capacity for nitrogen fixation and nitric oxide production, suggesting its capacity to promote plant growth. These findings offer significant insights into the banana microbiome and underscore its potential for fungal biocontrol and plant growth enhancement.

Keywords: *biocontrol, Fusarium, PGPR, banana Fusarium wilt, whole genome assembly*

Fusarium oxysporum f. sp cubense (Foc) is a fungal pathogen that invades banana roots and impedes water flow to the plant, debilitating nutrient acquisition and ultimately leading to its death. One of the most notable races of Foc is *Fusarium oxysporum f. sp cubense* Tropical Race 4 (FocTr4), which inflicted worldwide economic losses to export quality banana 'Cavendish', particularly in Australia and the tropical regions of Southeast Asia, including China, Indonesia, Malaysia, and the Philippines [1]. To preclude further economic losses, an effective strategy is to combat FocTR4 using biocontrol agents that can degrade the outer covering of FocTR4 and possibly its secreted proteins and metabolites [2]. Furthermore, a biocontrol that can improve nutrition while boosting the host plant's immune system would be an added benefit.

Endophytic and rhizospheric bacteria have been tapped as potential biological control agents. Endophytic bacteria live within the internal tissues of their host plant without causing disease [3]. Their colonizing capability inhibits the growth of Foc in an infected banana plant by producing antibiotics against pathogens [4]. Rhizospheric bacteria, on the other hand, form part of the population of microbes in the thin layer of soil that immediately surrounds the plant roots [5]. These bacteria benefit bananas as they provide essential nutrients and metabolites that promote plant growth and restore natural physicochemical characteristics of the soil while acting as an antagonistic agent against pathogenic microbes such as Foc [6–8]. With these attributes, endophytic and rhizospheric bacteria are potential candidates for a more robust biological control against Foc.

This research focuses on RS13, which was identified via the screening of endophytic and rhizosphere bacteria in bananas. This was carried out to discover Foc antagonists and bacteria that promote banana plant growth. Whole genome assembly of RS13 elucidated its taxonomic

classification and genomic features as a potential biocontrol agent and a plant growth-promoting bacterial isolate.

Materials and Methods

Isolation of Endophytic and Rhizospheric Bacteria from Banana Roots

For the isolation of putative endophytic bacteria, the methodology of Thangavelu & Gopi [9] was followed with some modifications. Basal pseudostem and rhizomes collected from a healthy Cavendish cultivar from Tboli, South Cotabato were initially cleaned by washing thoroughly with tap water to remove soil and other debris. Subsequently, portions (5 g) of these samples were separately immersed in 70% ethanol for 3 min, followed by washing with 1% (v/v) sodium hypochlorite solution for 5 min. These samples were then rinsed with 70% ethanol for 30 seconds and washed five times with sterile distilled water. Aliquots of the distilled water used in the final rinse were spread on Thermo Scientific™ Nutrient agar (NA) in Petri plates to confirm the successful sterilization process and to ascertain that the isolates were of endophytic origin.

For the isolation of rhizospheric bacteria, the methodology used by Gechamba et al [5] was followed with some modifications. Soil samples from the rhizosphere of a healthy Cavendish banana cultivar were collected from a depth of up to 20-40 cm in areas surrounding the banana plant roots. Soil particles adhering to the roots were scrubbed, then cleaned of debris, and 1gram of rhizosphere soil was diluted in 20 mL sterile 0.9% NaCl solution and shaken vigorously for 5 minutes. The solution was subsequently serially diluted to 10-3, inoculated on nutrient agar with 1mL Nystatin (15ppm) at room temperature, and observed for growth for 3 days. Morphologically distinct colonies were sub-cultured on NA slant and NA slant with mineral oil and maintained at 1-4°C until further use [10].

In Vitro Screening of Bacterial Isolates Against Foc

To detect the presence of antagonistic bacteria, the Visual agar plate assay of Tan et al. (2015) was followed. The *Fusarium oxysporum f. sp. cubense* conidial suspension was calibrated to an optical density (OD640) of Thermo Scientific™ 0.5 McFarland standard, and 20uL of the suspension was inoculated in a line at the center of Thermo Scientific™ PDA plates. The bacterial suspension of isolates was inoculated in the right and left lines 2 cm away from the central line, with *Escherichia coli* and *Bacillus subtilis* used as negative and positive controls, respectively. The plates were incubated at 26°C. Thereafter, the width of the upper, middle, and lower mycelial lines was measured every day until the 8th day [11].

Evaluation of Plant Growth-promoting Effects of Potential Isolates Under Nursery Conditions

Preparation of banana seedlings: One-month-old tissue cultured banana (cv. Cavendish) seedlings with 3-5 leaves were transplanted and were grown in plastic pots (15 x 15 x 10 cm) containing 2kg of solarized and autoclaved soil and sand (1:1 ratio)[11]. The pots were bagged doubly to prevent outflow contamination. Established banana plantlets were acclimatized for two weeks before testing.

To assess their plant growth-promoting potentials, the isolates were grown in Thermo Scientific™ nutrient broth for 48 h. The suspensions were allowed to grow in NA to confirm the CFU density of the isolates. The concentration was then adjusted to 5 CFU/mL using Thermo Scientific™ nutrient broth.

After acclimatization, the banana plants were treated with 100mL isolate suspension using a completely randomized design and utilizing these treatments: T0 or Mock Control (Potato dextrose broth); T1 or Positive Control (*Bacillus subtilis*); T2- RS12; T3- (ER03) and T4- ER20. Potted bananas were watered four times a week with 100mL water and were treated with 10mL commercial nutrient solution (NPK) twice a month.

The responses of the banana plants to the treatments were assessed through morphological parameters such as pseudostem diameter in cm (5cm above ground), pseudostem height (cm), number of fresh leaves, and leaf area (cm^2). Leaf area was calculated by taking photographs from a distance of 1.5m above the banana plantlet using Canon DS126431 with an EFS 18-55mm lens set in autofocus in manual mode. Using Adobe PS5, plant area was calculated using the formula:

$$\text{Leaf Area} = (L/C) \times 10\ cm^2;$$

wherein L is the total pixel occupied by the leaves in the photograph while C is the total pixels occupied by the control paper with the size of $10cm^2$ placed near the plantlets 15cm above the ground.

For the in vitro screening of isolates, the significance of the inhibition effect per day was tested with the one-way ANOVA method with Tukey post hoc analysis at a 5% confidence level. For the tests in the nursery, the significance of plant-growth-promoting effects was analyzed with a one-way ANOVA test with Tukey post hoc analysis at a 5% confidence level. Both statistical

analyses were carried out using the IBM SPSS statistics version 23, developed by the International Business Machines Corporation.

Whole Genome Sequencing and Assembly and Taxonomic Classification

DNA extraction and sequencing of RS13 were conducted at the Philippine Genome Center, University of the Philippines, Diliman. RS13 was grown in a nutrient broth and DNA was extracted using KingFisher Cell and Tissue DNA Kit. The library was prepared using Illumina Nextera XT with V3 as sequencing reagent and Illumina MiSeq Primary Sequence was run in 300 cycles.

Paired-end reads of RS13 were then assessed using FastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Poor-quality bases, low-quality reads with a Phred score lower than 30, short sequences with less than 20 bases, and sequencing adaptors were removed using TrimGalore v0.6.6. Thereafter, de-novo genome assembly was conducted using SPAdes V3.15.3, activating the error correction setting using the BayesHammer algorithm and removing low-quality bases through the '—careful' command.

Taxonomic classification of sequence reads, assembly optimization, genome visualization, contigs analysis, whole genome taxonomic classification, and functional annotation were conducted in Kbase [12], an open-source software and data platform for genomic analysis. Assembled reads were subsequently classified taxonomically through Kaiju [13]. To optimize the assembled genome from SPades, Unicycler v0.4.8 [14] was applied with a normal cut-off mode. Circular genome visualization was made through CGView while contigs were analyzed through Quast and viewed in the Icarus contig browser. Moreover, the GTDB-Tk v1.7.0 toolkit [15] was utilized to assign the taxonomic classification of the assembled genome.

In-silico Functional and Metabolic Analysis of RS13

To examine RS13 functional gene family distribution, gene prediction and functional annotation of the RS13 assembled genome were conducted through Prokka v1.14.5 [16]. Comparative analysis of functional and metabolic summary per genome was conducted between RS13 and *Morganella morganii* through SEED [17] and DRAM [18], respectively. The applications were run in Kbase [12]. The RS13 functional and metabolic profiles were then compared by examining SEED functional categories and DRAM metabolic categories. Functional categories exhibiting an increased number of genes for each metabolic category, along with the presence of genes in each category, were further linked to potential biocontrol and plant growth-promoting activities as supported by existing literature.

Further analyses of RS13 chitinases, LKFKLEPP_05769, LKFKLEPP_04415, and LKFKLEPP_04795 were likewise conducted. To screen for putative secreted RS13 chitinases, signal peptide prediction was conducted through DeepLoc2.0 [19]. To test for possible interaction between putative secreted chitinases of RS13 and Foc chitin, molecular docking was conducted between RS13 chitinases, LKFKLEPP_05769 and LKFKLEPP_04415, and Foc chitin in silico. Protein modeling of RS13 chitinases was conducted through AlphaFold [20], and molecular docking of chitinases against *Fusarium oxysporum* chitin was conducted through CB-Dock [21].

Results and Discussion

Isolation of Endophytic and Rhizospheric Bacteria from Musa sp. cv. Cavendish

A total of 67 morphologically differentiated colonies were isolated from three sources (i.e. Musa sp. Cavendish pseudostem, rhizome, and rhizosphere soil). Of this number, 70% were endophytic bacteria from rhizomes (27 isolates), and from the pseudostem of the banana plant (20 isolates) while the remaining 30% were isolated from the rhizosphere soil.

In Vitro Screening of Bacterial Isolates Against Foc

Out of 67 isolates, only 24 isolates showed unique cultural and morphological features in NA agar and survived after incubation in 28^{O}C. A total of 24 isolates were screened against Foc by placing the Foc mycelial line between two bacterial isolates, 2 cm from the Foc line. On day 2, the width of Foc started to become visible. Subsequently, from day 4 to day 8, significant differences in Foc TR4 mycelial width became even more apparent as the mycelia grew nearer to the bacterial isolate lines (Figure 1).

Foc was significantly inhibited by *B. subtilis*, RS13, ER03, and ER20 on days 5, 6, 7, and 8 compared to *E. coli*. There also were statistically significant inhibitions of Foc mycelial width in the presence of ER18 (day 5, 6), ER23 (day 5, 6, 7), ER27 (day 5, 6, 7) and EP03 (day 6, 7) compared to *E. coli*. In contrast, there were no significant differences between the antagonistic activities of RS13, ER03, and ER20 on Days 4, 5, 6, 7, and 8 compared to *B. subtilis*. Moreover, ER18 (day 4, 5, 6), ER23 (day 4,5,7, 8), and ER27 (day 4,5,7,8) likewise showed insignificant

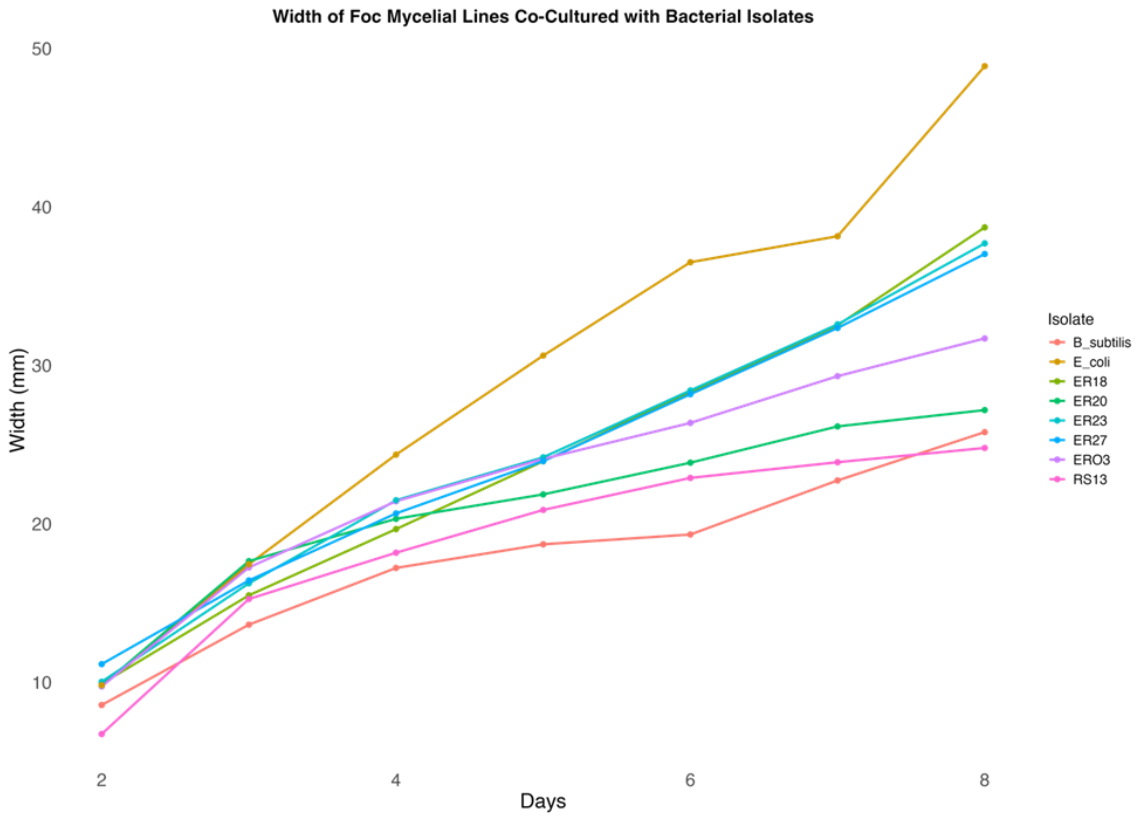

Figure 1. Visual agar plate assay. Foc mycelial mean width co-inoculated between isolates, 2-8days post incubation in nutrient agar.

differences in the antagonistic activities compared to *B. subtilis*. These showed the potential of the isolates as good candidates for biological control agents.

Effects of Bacterial Isolates on Banana Plantlets Under Nursery Conditions

Three bacterial isolates, RS13, ER03, and ER20, were chosen for evaluation of their plant-growth-promoting effects on banana plantlets under nursery conditions. Figure 2 illustrates the growth of banana plantlets at 90 days following inoculation. Figure 3 shows that plantlets inoculated with bacterial isolates had a mean pseudostem diameter trend of ER03>ER20>RS13. Compared to the negative control, plantlets inoculated with the bacterial isolates had wider pseudostem diameter, taller pseudostem height,

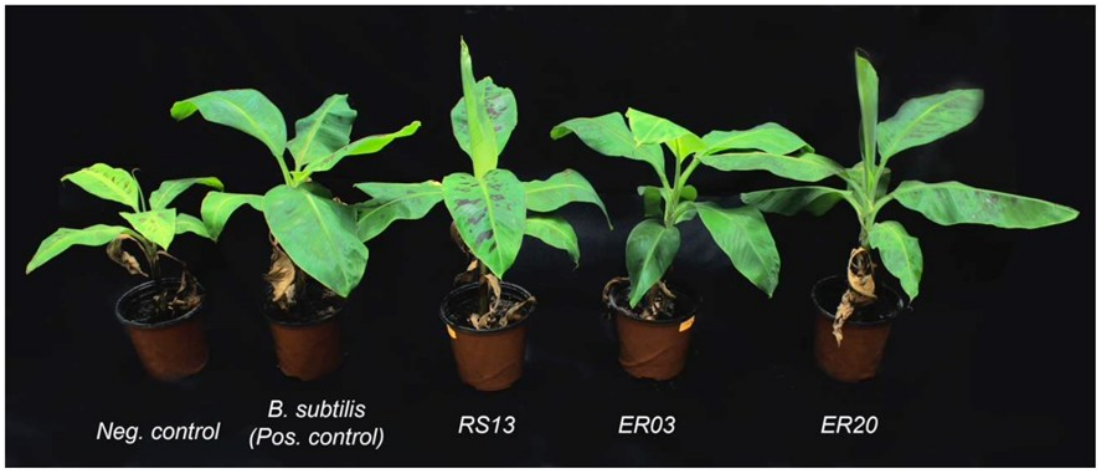

Figure 2. Cavendish banana plantlets growth at 90 days post inoculation on bacterial isolates.

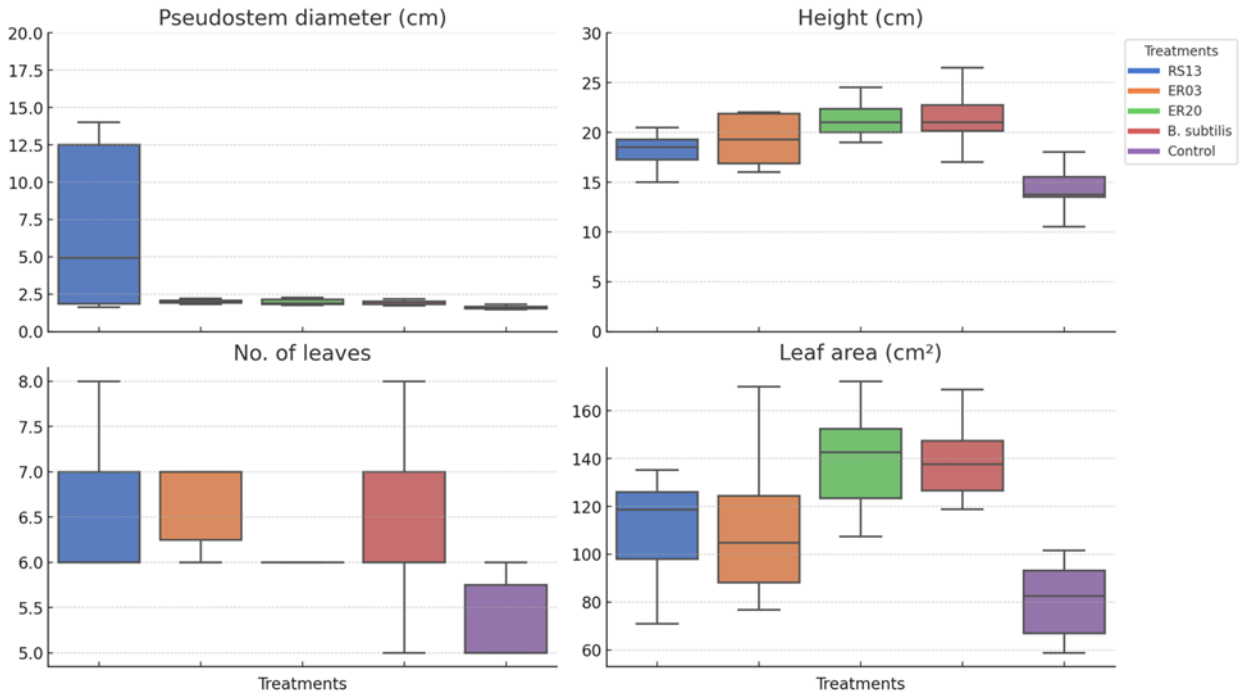

Figure 3. Plant-growth promoting effects of bacterial isolates RS13, ER03 and ER20 on banana plantlets 90 days post inoculation in terms of pseudostem diameter, height, leaf number and leaf area. Boxplot inside line represents median and whiskers indicate variability in the data.

increased number of leaves, and wider total leaf area. These effects were comparable to those of the positive control, *Bacillus subtilis*.

Optimized Whole Genome Assembly and Taxonomic Classification Show RS13 as Closely Related to *Morganella morganii subsp. morganii KT*

RS13 was chosen for whole genome analysis due to its better viability post-subculturing, in contrast to ER03 and ER20. A total of 1,088,388 reads per paired-end were generated from MiSeq sequencing of RS13. RS13 paired-end raw reads yielded 11Gb (>Q30) with an initial fastq analysis showing poor per base sequence quality for both fastq files (Phred scores between 6-38).

After trimming, R1 exhibited enhanced per-base sequence quality with a Phred score ranging from 20 to 38, whereas R2 continued to display poor reads with a Phred score range of 8 to 38. Table 1 illustrates the percentage of sequences filtered throughout the trimming process.

The de-novo assembly generated through SPades was optimized using Unicycler as shown in Table 2. The optimized assembly resulted in 30 scaffolds compared to 1613 scaffolds generated by SPades alone. The GC% and total length were improved from 44.87% to 50.06%, resulting in a total length of ~3.94M. The improved assembly of RS13 matched the overall length more closely and predicted the GC percentage of the genome of *Morganella morganii subsp. morganii KT*. This was further supported by mitochondrial protein analysis, where 87% of the reads were classified taxonomically as belonging to *Morganella morganii*. More than 10% of the reads, however, were unclassified.

Further taxonomic classification was

Table 1. Percentage of RS13 filtered sequences.

Dataset	Total base pairs processed	Quality trimmed	Total written (filtered)
R1 1,088,388 reads	278,657,169 bp	34,482,351 bp (12.4%)	243,557,098 bp (87.4%)
R2 1,088,388 reads	290,033,934 bp	137,159,674 bp (47.3%)	152,258,641 bp (52.5%)

conducted by aligning 49 gene families of RS13 to reference genomes using GTDB-Tk. RS13 was classified under the phylum Proteobacteria, class Gammaproteobacteria, order Enterobacterales, family Enterobacteriaceae, and the genus *Morganella*. However, the genome was not assigned to the closest species as it fell outside its pre-defined average nucleotide identity (ANI) radius compared to *Morganella morganii.* Furthermore, phylogenome analysis conducted using the Kbase tree revealed RS13 to be closely related to *Morganella morganii subsp. morganii* KT (Figure 4B).

In the SPades assembly, 5,894 genes were predicted of which 5,776 were forecasted to be protein-coding genes. Additionally, a total of 3,607 genes were determined to have non-hypothetical functions. The average protein length was discovered to be 303 amino acids. In the optimized assembly with the Unicycler, 3,745 genes were identified, of which 3,668 were predicted to be protein-coding. Additionally, 2,563 genes were identified as having no hypothetical function, with an average protein length measuring 310 amino acids.

Comparative Functional and Metabolic Analysis of RS13 Reveals Putative Genes Involved in Biocontrol and Plant Growth-promoting Activity

To identify important genes involved in the biocontrol and plant-growth-promoting activity of RS13, the optimized RS13 assembly was functionally annotated using SEED and compared with *M. morganii*. RS13 was shown to have an increased number of proteins in some gene families involved in the Type III secretion system - invasion protein InvA, surface presentation of antigens - spaP and spaQ, and Trk system potassium uptake protein - TrkG, compared to the reference genome, *M. morganii subsp. morganii KT* (Figure 4A). These gene families might be important in the secretion of hydrolytic enzymes against *Fusarium* and nutrient acquisition that will ensure the survival of host plants.

Table 2. Genome assembly of RS13 using SPades and Unicycler compared to *Morganella morganii*

Genome assembly parameters	*M. morganii* subsp. morganii KT	RS13- SPades	RS13 Spades + Unicycler
No. of scaffolds (≥ 500 bp)	1	1613	30
Largest scaffold (bp)	3 799 539	1 089 774	758 254
Total length (bp)	3 799 539	6 838 443	3 936 838
N50	3 799 539	218 219	252 545
GC (%)	51.14	44.87	50.06
Mismatches (N), N's per 100 kbp	5, 0.13	100, 1.46	0, 0
PROKKA Predicted genes	3 626	5 894	3 745

The metabolic profile showed that RS13 could potentially enhance nitrogen metabolism (such as the production of nitrate, nitrite, and nitric oxide), sulfur metabolism (such as the production of thiosulfate), methanogenesis (or methane production), and alcohol conversion through conversion of pyruvate to acetyl CoA and producing acetate, lactate D, lactate L, and other alcohols. Additionally, gene mining revealed that RS13 has genes involved in chitin, glucan, and cellulose degradation. Among the chitinases found, LKFKLEPP_04415 showed an extracellular signal peptide (0.57) and a strong binding affinity (-8.5 kcal/mol) for Foc chitin (Figure 4C). These functional predictions can provide strong bases for RS13's antifungal and plant growth-promoting activity.

This study employed whole genome analysis to taxonomically classify and determine the potential basis of RS13's antagonistic activity against *Fusarium* spp. and its growth-promoting activity in banana plants. Whole genome sequencing for RS13 was carried out in MiSEQ, which generated short reads. While initial assembly through SPades resulted in an assembly with fragmented contigs, this was improved using Unicycler, resulting in fewer contigs and fewer misassemblies, as identified in QUAST. These outcomes can be attributed to the capacity of Unicycler to correct sequencing errors, choose the ideal maximum k-mer, eliminate other repeats between single copy contigs, and align the final assembly [14]. This suggests that the further

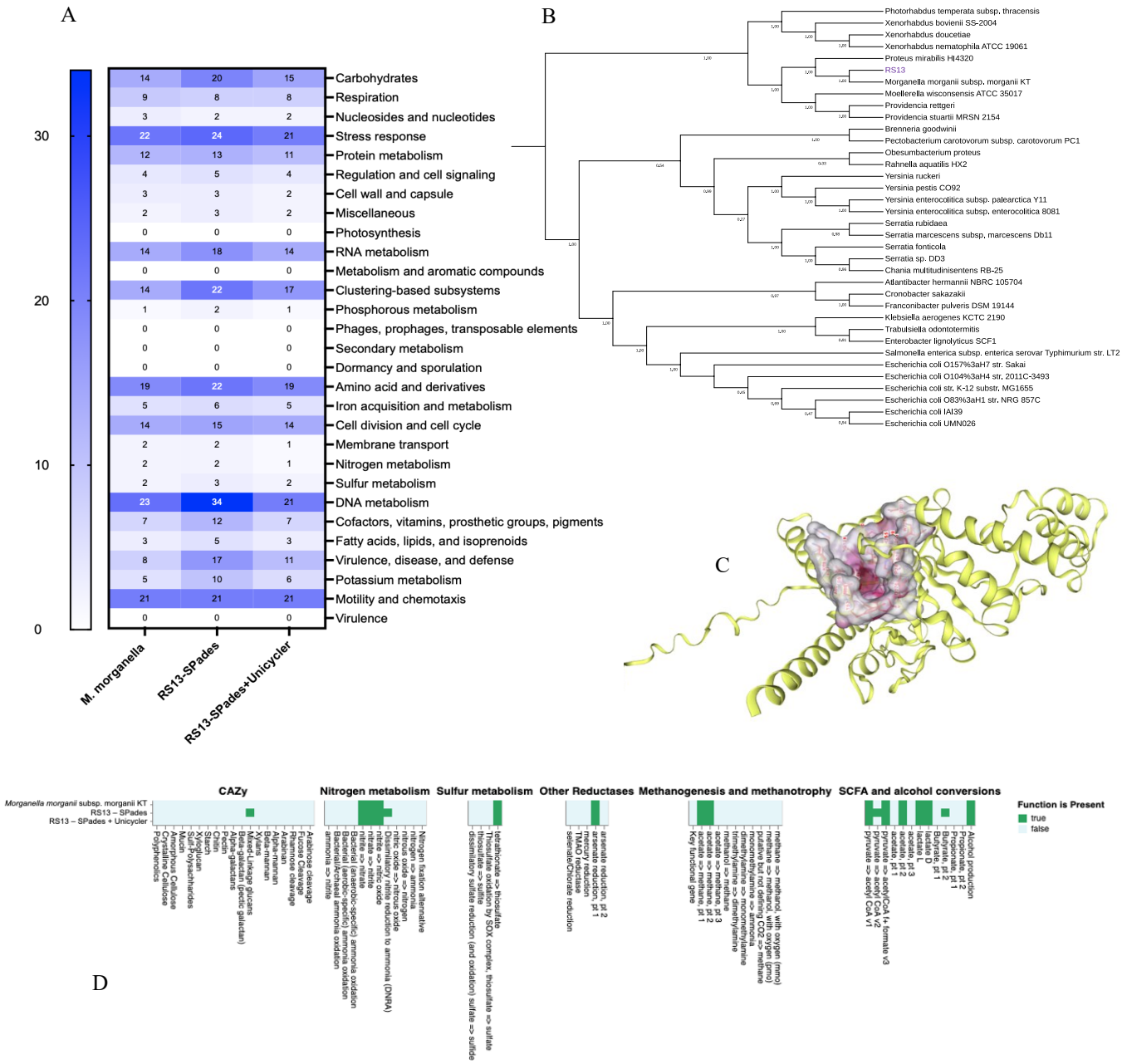

Figure 4. Whole genome identification, functional annotation of RS13. **A**. SEED Annotation of RS13 genes showing relative similarity of optimized genome (RS13- SPades + Unicycler) to *Morganella morganii subsp. morganii* KT. **B**. Phylogenomic Tree if RS13 with boostraps compared to 35 subsets of KBase genome that were closely related to RS13 using neighbor public genome count. Multiple sequence alignment was conducted for 49 COG (Clusters of Orthologous Groups) gene families through FastTree2. **C**. Molecular docking of chitinase LKFKLEPP_04415 showing a strong binding affinity to substrate chitin in the putative active site. **D.** Comparative metabolic profile of RS13 genome assemblies compared to SPades inferred through DRAM.

identification and functional annotation of RS13 have improved because of the optimized genome.

In the three-step taxonomic classification through Kaiju sequence in comparison to the reference database of microbial proteins, GTDB-Tk classification through bacterial marker genes, and Kbase tree phylogenomic analyses of core gene families of RS13, whole genome assembly through Unicycler identified RS13 as *Morganella morganii*. Whole genome assembly through SPades alone failed to classify RS13 even at the genus level. This shows that Unicyler can also improve the taxonomic classification of assembled genomes. After all, the classification of RS13 remains

unresolved at the species level since it falls outside its pre-defined average nucleotide identity (ANI) radius compared to *Morganella morganii* and the whole genome assembly showed numerous contigs. It can thus be inferred that the precision of RS13 taxonomy classification will be more accurate with long-read sequencing and substantial reference genome coverage. Moreover, the functional annotation of RS13 indicated that, regarding the electron transport chain protein components and metabolic profile, RS13 exhibits a comparable profile with few unique proteins to *Morganella morganii subsp. morganii KT*.

Furthermore, RS13, a naturally occurring microorganism in rhizosphere soil, showed strong potential as a biocontrol agent against Foc. Biocontrols targeting Foc possess the ability to induce resistance in plant hosts, exhibit antibiosis, and promote plant promotion [22]. Whole genome analysis of RS13 provided evidence for these functional characteristics.

RS13 may induce resistance in bananas against Foc. It is theorized that when bananas detect Foc TR4, they produce jasmonic acid, which signals TR4 to inflict nitrosative stress on bananas through nitric oxide bursting. Due to low members of enzymes that could detoxify nitric oxide, bananas become vulnerable to nitrosative stress [23]. To mitigate this, RS13, with its innate capacity to produce nitric oxide, serves as a pre-emptive inoculum to activate the banana's nitric oxide detoxification mechanism before TR4 infection occurs. The stimulation of the nitric oxide detoxification mechanism has been shown to enhance banana tolerance against Foc using nitric oxide donors [24].

On the other hand, the antibiosis capacity of RS13 can be attributed to its increased members involved in the secretion system and the availability of secreted chitinases. The type III secretion system, including invasion protein, InvA; surface presentation of antigens proteins spaP and spaQ were found to have two members in RS13 compared to single members in *M. morganii subsp. morganii* KT. The type III secretion system proteins are essential for eukaryotic host invasion and immunity modulation [25]. In *Lysobacter* spp., type III secretion systems serve as conduits for hydrolytic enzymes inhibiting fungal growth [26], [27]. Moreover, this secretion system is believed to secrete antimicrobial compounds that can interfere with pathogen virulence [28]. Additional gene mining showed that RS13 has three chitinases, playing a crucial role in the degradation of the fungal exoskeleton and impairment of quorum sensing that reduces pathogen infection and symptoms of plant diseases [29]. These chitinases target the extracellular exoskeleton of fungi, produced in huge quantities by *Fusarium* species as N-acetylglucosamine [30]. Among the three chitinases, LKFKLEPP_04415 is predicted to be secreted extracellularly by RS13, and its strong binding to chitin through molecular docking indicates its putative activity against fungal chitin of *Fusarium oxysporum.*

The plant growth-promoting capacity of RS13 could be attributed to its capability for nitrogen fixation. Specifically, RS13 can metabolize nitrite to nitrate — the readily absorbed form of nitrogen in plants. As a rhizosphere soil bacterium, RS13 is expected to abound in areas surrounding plant roots, where it creates nitrate that the plants can readily absorb. With few biocontrol agents sourced directly from rhizosphere soil [31], RS13 could serve as an additional rhizosphere-sourced bioinoculant, enhancing the diversity of beneficial microbes that can be incorporated into the planting medium during and after micropropagation.

RS13, through its two-fold benefits (as a biocontrol agent and a plant growth promoter), may benefit banana productivity especially when grown in a plantation setting characterized by heavy fungicide use. It may significantly reduce Fusarium disease incidence while enhancing plant growth, improving soil conditions, reducing human exposure to harmful fungicides and providing an environmentally friendly alternative to chemical sprays. Through these benefits, the country will come closer to the attainment of its goals of food security, human health and well-being, and the protection of the environment.

Conclusion and Recommendations

RS13 exhibits strong potential as a biocontrol agent against *Fusarium oxysporum f. sp. cubense*. Whole genome analysis supports its attributes to suppress Foc, induce immune tolerance and promote growth in banana. The identification of chitinases and type III secretion system proteins in RS13 highlights possible mechanisms underlying its antifungal activity. Further validation through knockout experiments and heterologous expression studies will be crucial in confirming their role in disease resistance. Additionally, RS13's predicted capability for nitrogen fixation and nitric oxide production may play a significant role in promoting banana growth and strengthening immune responses in banana against Foc. These findings support RS13's potential for integration into biocontrol strategies for managing Fusarium wilt in bananas, thereby contributing to the current body of knowledge about sustainable methods for Fusarium control and plant growth promotion.

Author's Contribution

JC designed the study, carried out the experiments, analyzed the data, and prepared the manuscript. DA designed and edited the manuscript.

Data availability

RS13 whole genome sequence could be accessed through Sequence Read Archive – National Center for Biotechnology Information (SRA-NCBI) with accession number PRJNA1201792.

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