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Biohardening of Banana cv. Karpooravalli (ABB; Pisang Awak) With *Bacillus velezensis* YEBBR6 Promotes Plant Growth and Reprograms the Innate Immune Response Against *Fusarium oxysporum* f.sp. *cubense*

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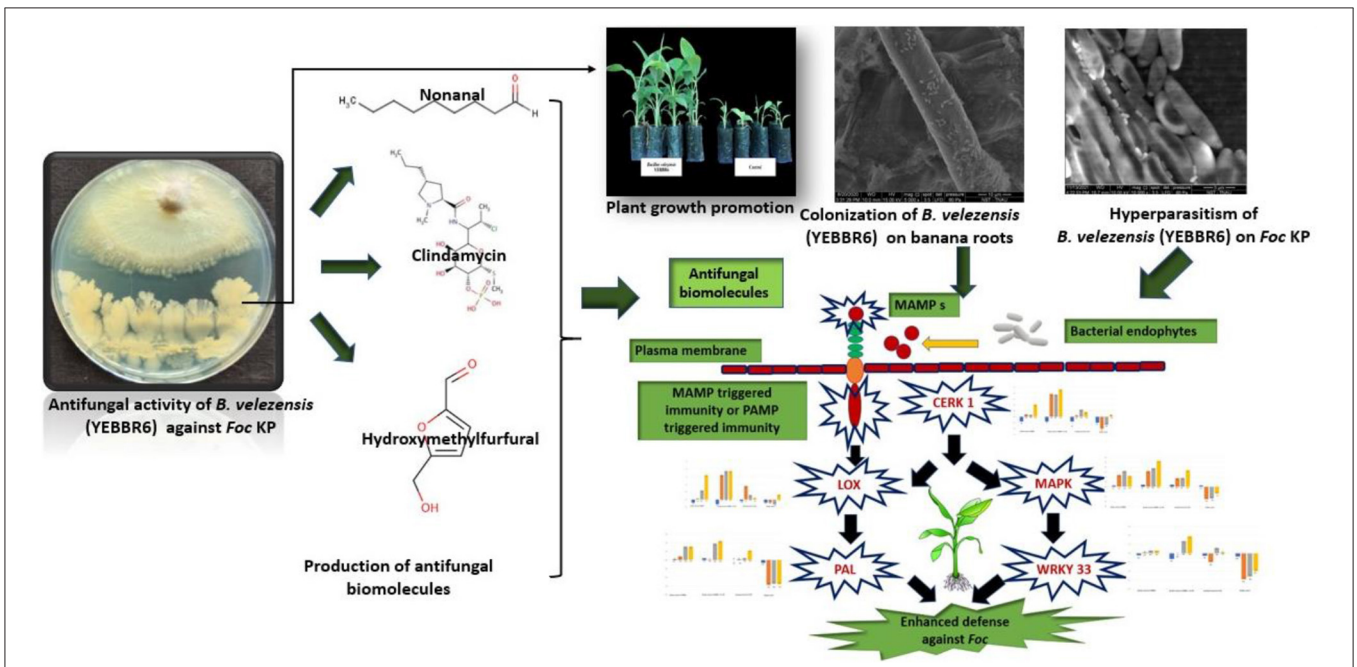
Fungicides play an immense role in quenching the infection of Panama wilt in banana. However, the use of fungicides and the monoculture of banana cultivars have resulted in the development of new races like race 4 which challenges scientists across the globe to identify new candidates for biological suppression of *Fusarium oxysporum* f.sp. *cubense* (*Foc*). Hence, attempts were made to dissect the endophytes from resistant banana cultivar YKM5 (Yengambi–AAA) to suppress *Foc* KP (race 4) infecting cv. Karpooravalli (ABB; Pisang Awak). Among the various endophytes, *Bacillus velezensis* YEBBR6 inhibited the mycelial growth up to 63% over control and hyper-parasitized the mycelium of *Foc* KP. Scanning electron microscope analysis revealed the ramification by *B. velezensis* over the hyphae of *Foc* KP leading to lysis. Analysis of VOCs/NVOCs compounds from the zone of inhibition, confirmed the presence of unique biomolecules including linoelaedic acid, nonanol, acetylvaleryl, 5–hydroxyl methyl furfural, clindamycin, allobarbital, 3-thiazolidine carboxamide, azulene, aminomorpholine, procyclidine, campholic acid, 3 amino-4 hydroxy phenyl sulfone, 3–deoxy mannoic lactone, hexadecanoic acid, oleic acid, and dihydroacridine of an antifungal and antimicrobial nature. Considering the diverse antimicrobial property, biohardening of micropropagated banana cv. Karpooravalli (ABB) with a liquid formulation of *B. velezensis* YEBBR6 (8×10^8 cfu/ml) and challenge inoculation with *Foc* KP promoted plant growth compared to uninoculated control. Besides, incidence of *Fusarium* wilt was reduced by 100% over inoculated control in greenhouse conditions. Furthermore, the expression of transcription factors and defense genes WRKY, MAPK, CERK 1, LOX, and PAL increased by several folds compared to inoculated and healthy control and thus suppressed *Fusarium* wilt of banana cv. Karpooravalli (ABB). Also, cytoscape analysis of defense

genes indicated the coordinated expression of various other genes associated with it. Hence, our study confirmed the scope for exploring *B. velezensis* on a commercial scale for the management of *Fusarium* wilt race 4 causing wilt across genomes of banana.

Keywords: banana, *Fusarium* wilt, *Bacillus velezensis*, biohardening, VOCs/NVOCs compounds, defense genes expression

INTRODUCTION

Globally, banana is commercially cultivated in several tropical and subtropical regions. Monoculture of banana has resulted in the outbreak of *Fusarium* wilt. Panama wilt of banana caused by *Fusarium oxysporum* f.sp. *cubense* (*Foc)* is a dreadful disease affecting the livelihood of the farming community and the sustainability of banana-based industries. Pathogens survive as chlamydospores in soil for decades. Furthermore, owing to the proliferation of pathogens, outbreak of race 4 has emerged as a most destructive pathogen worldwide (Butler, 2013). Race 4 was observed in 90% of banana plantations in South China (Cheng et al., 2012). Recently in India, *Foc* TR4 has been reported in Gujarat and Bihar, indicating the potential spread and threat in the near future (Nakkeeran et al., 2021). Hence, to date there are no effective fungicides to curb the spread and infection of banana by race 4. This lacuna warrants the development of a novel management strategy to quench *Fusarium* wilt of banana. Besides, one school of thought also emphasizes that continuous application of chemical fertilizers acidifies the soil and accentuates the multiplication of *Foc* resulting in severe outbreaks of *Fusarium* wilt (Zhao et al., 2018). Considering the serious threat imposed by *Foc*, an effective alternate method has to be developed for the management of *Fusarium* wilt of banana. To reach sustainability and profitability in food production, plant growth-promoting rhizobacteria (PGPR) play a key role and also aid in increasing the productivity of plants both under biotic and abiotic stress. Furthermore, PGPR suppress plant pathogens through competition, antibiosis, lysis, and through induction of systemic resistance (Anupama et al., 2014; Narasimha Murthy et al., 2021). In this juncture, biological control will pave the way for the sustainable management of wilt caused by different races of *Foc*. Amidst the various biocontrol agents, bacterial endophytes can be well explored for the same, rather than fungicides (Nel et al., 2006). Several endophytic *Bacillus* spp. have been explored for the management of *Fusarium* wilt of banana as they are bestowed with beneficial attributes including plant growth promotion, induction of immune response, and suppression of *Foc* (Nakkeeran et al., 2021; Saravanan et al., 2021a). In the recent past, *Bacillus* spp. have been well explored for the management of soil-borne and foliar diseases. The antagonistic *Bacillus* spp. quench plant pathogens through the



GRAPHICAL ABSTRACT | Induction of innate immune response and growth promotion in banana by *B. velezensis* against Foc.

production of antifungal biomolecules, antimicrobial peptides, and through the induction of an immune response (Nakkeeran et al., 2019). However, the versatile *Bacillus* spp. genome of *B. velezensis* comprises genes coding for different antimicrobial peptides, hydrolytic enzymes, growth hormones, and induction of immune response contributing to antiviral and antifungal action (Saravanan et al., 2021b). Moreover, *B. velezensis* has broad-spectrum action against several fungal pathogens (Meng and Hao, 2017). However, based on the perusal of literature there are no reports describing the exploration of immune response mediated by *B. velezensis* to manage *Foc* infection in banana. In the current investigation, *B. velezensis* YEBBR6 isolated from the bract of resistant genotype YKM 5 was evaluated for antifungal activity, growth promotion, secondary metabolite production, and reprogramming of immune response for the management of *Fusarium* wilt of banana through biohardening of micropropagated cv. Karpooravalli (Pisang Awak–ABB) plantlets.

MATERIALS AND METHODS

Isolation and Molecular Confirmation of *Fusarium oxysporum* f. sp. *cubense*

The wilt affected banana corms from different banana-cultivating provinces of Tamil Nadu, India pertaining to Lakshmipuram village of Theni province (10° 11′ 16.62″ N77° 47′ 34.4112″E/ latitude 10.187950/ longitude 77.792892), Chinnamanur village of Theni province (9° 50′ 22.776″N77° 22′ 58.0692″E/ latitude 9.839660/ longitude 77.382797), Thondamuthur village of Coimbatore province (11°00′35″N 76°49′41″E/ latitude 10.9905/ longitude 10.9905), Sirumugai village of Coimbatore province (11° 19′ 16.9″N, 77° 0′ 18.8″E/ latitude 11.3183/ longitude 77.0066), Ammapalayam village of Salem province (11° 40.7123′ N 78° 7.4319′ E/ latitude 11.678539/ longitude 78.123865), Athani village of Erode province (11.5232° N, 77.5120° E/ latitude 11.515219/ longitude 77.452367), Andhiyur village of Erode province (11° 34′ 37.4628″N 77° 35′ 15.8280″E/ latitude 11.577073/ longitude 77.587730), and Gobichettipalayam village of Erode province (11.4548° N, 77.4365° E/ latitude 11.450410/ longitude 77.430036) were collected. Pathogenic *Fusarium* spp. associated with banana cultivars susceptible to Panama wilt of banana were isolated as per the protocol described by Nelson et al. (1983). Genomic DNA of *Fusarium* was extracted from the mycelium of pure culture through the CTAB method (Griffith and Shaw, 1998). Genomic DNA was used as a template for PCR amplification of *Foc* isolates using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers (White et al., 1990). Further to confirm the *Foc* races, secreted in Xylem (SIX) Six13c 343 F (5′CAGCCTCCTAGCGTCGAAAA 3′) and Six13c R (5′CCGTGATGGGGTACGTTGTA 3′) were used (Czislowski et al., 2018). The program cycle comprised of initial denaturation (95°C) for 2 min, followed by 40 cycles of denaturation (95°C) for 1 min, annealing at 58°C for 1 min, extension for 1 min at 72°C, and with a final extension at 72°C. Gel electrophoresis and staining was done by loading 10 μl of PCR product on 1% agarose gel in TAE buffer at 80 V for 50 min at 25°C. A 1 kb DNA ladder was used to determine the size of amplified genomic products. PCR products were photographed using a gel documentation system. The amplified genomic product was sequenced by Eurofins Genomics Biotech Pvt. Ltd., Bangalore, India. Gene homology searches were performed using NCBI BLAST. Sequences were compared with different *Foc* isolates retrieved from the GenBank database. Newly obtained sequences were submitted to the GenBank database (New York, USA) and accession numbers were obtained. Phylogenetic analysis was performed with MEGA7 software (Tamura et al., 2007).

Isolation and Characterization of Bacterial Endophytes From Banana cv. Yengambi KM5 (AAA)

An antagonistic bacterial endophyte was isolated from 11-month-old *Foc*-resistant banana cv. Yengambi KM5 (AAA) maintained at the Banana Field Gene Bank, Tamil Nadu Agricultural University, Coimbatore (latitude: 11° 07′ 3.36" N, longitude: 76° 59′ 39.91" E), Tamil Nadu, India. The identity of antagonistic bacterial endophytes against *Foc* KP was confirmed using a 1.5 kb full-length 16S rRNA gene: 8 F (5′AGAGTT TGATCCTGGCTCAG-3′), 1492 R (5′GGGTTACCTTG TTACGACTT-3′). Antagonistic isolates YEBBR6, YEBN2, YEBRH5, YEBRT4, YEBFR1, and YEBFL6 that inhibited *Foc* KP were confirmed as *Bacillus velezensis* (MT372157), *Bacillus albus* (MT120179), *Achromobacter xylosoxidans* (MK258170), *Beijerinckia fluminensis* (MK263670), *Acinetobacter refrigeratoris* (MT326234), and *Bacillus endophyticus* (MT326238), respectively (Saravanan et al., 2021a).

GC/MS Analysis of Volatile Organic Compounds and Non-volatile Organic Compounds Extracted From the Zone of Inhibition of *Foc* KP and *B. velezensis* YEBBR6

The efficacy of bacterial endophyte *B. velezensis* YEBBR6, isolated from resistant banana cv. Yengambi KM5 (AAA) was tested *in vitro* against *F. oxysporum* f. sp. *cubense Foc* KP (NCBI accession no. MW 436477). The ability of the antagonist *B. velezensis* to suppress *Foc* KP was assessed through a dual culture technique. A 9 mm mycelial disc was excised using a sterile cork borer from a 7-day-old culture of *Foc* KP. It was placed on one side of a Petri plate containing PDA medium, 10 mm away from the periphery. The bacterial endophyte (24 h old) was streaked on the medium 10 mm away from the periphery, exactly opposite the mycelial disc of *Foc* KP. The plates were incubated at 28 ± 2°C for 7 days. The VOCs/NVOCs produced by *B. velezensis* YEBBR6 in PDA medium from the zone of inhibition were extracted by excising the agar from the zone of inhibition using a sterile scalpel. Excised agar with VOCs/NVOCs was blended with HPLC-grade acetonitrile in 1:4 ratios (5 g agar in 20 ml of HPLC grade acetonitrile). The mixture was sonicated twice for 30 s at 30% of the power of the sonicator for homogenization. After homogenization, samples were centrifuged and filtered to remove solid particles. The samples were dried in a vacuum

flash evaporator (Rotrva Equitron Make). After removing the eluent, the final product was dissolved in 1 ml of HPLC-grade methanol (Cawoy et al., 2015). The difference in VOCs/NVOCs profile produced during the interaction of *B. velezensis* YEBBR6 with *Foc* KP was characterized with PDA control, pathogen-inoculated control and bacterial antagonist-inoculated control through GC/MS (GC Clarus 500 Perkin Elmer Analysis) using the NIST version 2005 MS data library.

Development of Liquid Formulation of Antagonistic Bacterial Endophytes

Liquid formulation was developed by inoculating single colonies of *B. velezensis* YEBBR6, *B. albus* YEBN2, *Achromobacter xylosoxidans* YEBRH5, *Beijerinckia fluminensis* YEBRT4, *Acinetobacter refrigeratoris* YEBFR1, and *Bacillus endophyticus* YEBFL6 in Luria Bertani (LB) broth. Inoculated LB broth was incubated at 28 ± 2°C for 72 h in an orbital shaker at 150 rpm. The concentration of bacterial cells in the culture broth was assessed by measuring the absorbance at 600 nm in a bio-spectrophotometer (Eppendorf Make) at an optical density (OD) of 1.0 ABS. Later, bacterial suspension in LB broth pertaining to six different bacterial antagonists was blended with 10 ml of glycerol (1%), 10 ml of tween 20 (1%), and 1% poly vinylpyrrolidone (PVP) supplied from Sigma–Aldrich. The resultant mixture of individual bacterial antagonists was mixed by incubation in an orbital shaker at 150 rpm for 10 min. The formulation of individual bacterial antagonists was adjusted to 8×10^8 cfu/ml (Vinodkumar et al., 2017).

Biohardening of Micropropagated Banana cv. Karpooravalli (ABB) Plantlets With a Liquid Formulation of Antagonistic Bacterial Endophytes

Micropropagated banana cv. Karpooravalli (ABB) plantlets were biohardened with a liquid formulation of antagonistic bacterial endophytes (8×10^8 cfu/ml). Micropropagated plantlets maintained in the protrays were biohardened on days 15 and 30 by drenching the root zone with 1% bacterial formulation. Biohardened plantlets were planted in polybags filled with EC (< 0.6 mS / cm) and pH (7)-stabilized sterile Cocopeat and incubated in the mist chamber. After 1 month of primary hardening, the micropropagated plantlets were subjected to secondary hardening. During secondary hardening, banana plantlets were transplanted in polybags (10 cm x 15 cm) containing one part of sterile red soil, one part of sterile sand, and one part of sterile farmyard manure mixture. The plantlets were hardened twice at 15-day intervals with 1% liquid formulation of antagonistic bacterial endophytes (8×10^8 cfu/ml) in the root zone until saturation and maintained in a greenhouse for further studies. Micropropagated banana cv. Karpooravalli (ABB) plantlets drenched with water served as untreated control.

The photosynthetic rate (μmol CO_2 m^{-2} s^{-1}), transpiration rate (mmol H_2O m^{-2} s^{-1}), stomatal conductance (m mol $m^{-2}s$), chlorophyll stability index, and relative water content (%) were recorded on the fully expanded leaves of banana plantlets using the LI-COR (LI-6400XT) Portable Photosynthesis System (PPS). The leaf chamber was the open type and measurements were taken at 10:00 h (IST). While taking measurements, photosynthetically active radiation of 1,500 μmol m^{-2} s^{-1} was maintained with an inbuilt light source. A temperature of 25 ± 5°C, relative humidity of 65 ± 5%, and reference carbon dioxide at a concentration of 380 mol CO_2 mol air^{-1} were also maintained. Observations were recorded after the plant reached steady-state photosynthesis (15 days after transferring to the greenhouse).

Evaluation of Micropropagated Banana cv. Karpooravalli (ABB) Plantlets Biohardened With Antagonistic Bacterial Endophytes Against *Foc* KP in a Greenhouse

The experiment consisted of six treatments, *viz., B. velezensis* YEBBR6, *B. albus* YEBN2, *Achromobacter xylosoxidans* YEBRH5, *Beijerinckia fluminensis* YEBRT4, *Acinetobacter refrigeratoris* YEBFR1, and *Bacillus endophyticus* YEBFL6, replicated three times with 10 plantlets per replication. Inoculum of *Foc* KP was multiplied in quarter-strength Potato Dextrose Broth (PDB) incubated for 7 days at 28 ± 2°C. Conidial load in the culture broth was assessed using a hemocytometer. Concentration of conidia was adjusted to 1×10^6 spores per ml (Catambacan and Cumagun, 2021). Biohardened micropropagated banana cv. Karpooravalli (ABB; Pisang Awak) plantlets with different bacterial endophytes were challenged with conidial suspension of *Foc* KP. Simultaneously, untreated control was also maintained. Banana plantlets biohardened with different bacterial endophytes challenged with *Foc* KP were watered regularly until saturation of soil moisture. Eight weeks after inoculation of *Foc* KP, the degree of disease severity was assessed. It was assessed based on leaf yellowing using a modified disease rating scale (Dita et al., 2014). The intensity of rhizome discoloration was scored by examining the longitudinal section of the pseudostem to the rhizome using a modified rating scale (Carlier et al., 2003).

Scoring for External Symptoms

S. No	Symptom description	Scale
1.	No symptoms	0
2.	Yellowing of lower leaves at initial stage	1
3.	Yellowing of all the lower leaves with discoloration of younger leaves	2
4.	All leaves with intense yellowing or plant dead	3

Scoring for Internal Rhizome Symptoms

S. No	Symptom description	Scale
1.	No symptoms	0
2.	Initial rhizome discoloration (1–20%)	1
3.	Slight rhizome discoloration along with the discoloration of the whole vascular system (21–40%)	2
4.	Rhizome with most of the internal tissues expressing necrosis (> 40%)	3

Percent disease severity was calculated as:

$$\text{Percent disease severity} = \sum \frac{\text{Number of plants in a specific scale category} \times \text{specific scale category}}{\text{Total number of samples} \times \text{maximum scale category}} \times \boxed{100}$$

Colonization of Rhizoplane by *B. velezensis* YEBBR6 and *Foc* KP in Biohardened Micropropagated Banana cv. Karpooravalli (ABB) Documented Through Scanning Electron Microscopy

One centimeter of banana root bits were fixed in 2.5% glutaraldehyde and 2.5% formaldehyde prepared in 0.1 M phosphate buffer of pH 7.2 (Liu et al., 2014) for 24 h at 4°C. Samples were exposed to a mild vacuum so as to ensure rapid infiltration of the fixative into the specimens. The fixed specimens were washed three times with 0.1 M phosphate buffer (pH 7.2) by incubation at 23°C for 30 min each time. The specimens were infiltrated overnight with 30% glycerol in water as a cryoprotectant. Later, specimens were frozen in liquid nitrogen, then cross-sectioned with a sterile scalpel. Later, specimens were post-fixed in 1% OsO4 in 0.1 M phosphate buffer (pH 7.2) for 1 h. Subsequently, samples were washed three times in deionized water and incubated for 30 min for each wash. Next, specimens were dehydrated in 100% ethanol and air-dried to remove the ethanol residues. The dried specimens were mounted on aluminum stubs and sputter-coated with gold. SEM photographs pertaining to colonization of the rhizoplane by *B. velezensis* YEBBR6 and *Foc* KP and hyperparasitic interaction of *B. velezensis* YEBBR6 on *Foc* KP were documented with Hitachi S-3500N 143 SEM at 15 kV.

Assessment of Defense Gene Expression in Micropropagated Banana cv. Karpooravalli (ABB) Plantlets During the Interaction of *B. velezensis* YEBBR6 and *Foc* KP Through qRT-PCR

Total RNA was extracted from the roots of banana biohardened with *B. velezensis* YEBBR6 challenged with *Foc* KP using Trizol (Sigma Aldrich) at 0 h, 24 h, 48 h, and 72 h after *Foc* KP inoculation (Chomczynski and Sacchi, 1987). Similarly, RNA was extracted from untreated healthy control, *B. velezensis* YEBBR6 alone, and *Foc* KP-inoculated control. RNA extracted from different treatments was made up to 3,000 ng/l using Nanodrop (Eppendorf Make, Germany). According to the manufacturer's protocol, respective RNA from different treatments was digested using DNase I (Sigma Aldrich, USA). Further, the quality of RNA was determined by measuring the absorbance value at an A260/A280 ratio. RNA was converted to cDNA with the ThermoFischer Scientific-RevertAid First Strand cDNA Synthesis Kit (cat. # K1622). A ratio of 1.8 + 0.2 indicated the best quality of nucleic acid. The cDNA was diluted 10-fold and used for qRT-PCR analysis. It was performed in a BIO-RAD CFX manager system. The reaction mixture for qRT-PCR comprised 3 μl of cDNA template, 10 μl of SYBR Green master mix (KAPA SYBR@FAST for LightCycler 480, Cat-KK4610), 0.8 μl of 10 μM forward primer, and 0.8 μl of 10 μM reverse primer. The final volume was made up to 20 μl using nuclease free water. The PCR program included denaturation at 95°C for 10 min, amplification for 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. It was followed by standard melting temperature analysis. The major defense gene transcripts assessed for the induction of resistance response against *Foc* KP infection were WRKY33, mitogen-activated protein kinase (MAPK), chitin elicitor receptor kinase (CERK 1), lipoxygenase (LOX), and phenylalanine ammonia lyase (PAL). For each defense gene expression study, three biological replicates and two technical replicates were maintained throughout the study. The fold changes in gene expression were calculated using the formula $\Delta\Delta Ct = \Delta$ Ct sample– Δ Ct reference. The relative fold changes in the transcript level were represented graphically by converting the $\Delta\Delta$CT value to $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Statistical analysis for relative fold change was performed using TIBCO Spotfire Analyst version 7.11.1.

Protein-Protein Interaction Analysis

Defense-related proteins in banana cv. Karpooravalli (ABB) plantlets that had a predominant role against *Foc* KP infection were further investigated for their interacting partners using the STRING database (Szklarczyk et al., 2021). Protein sequences of WRKY33, MAPK, CERK1, PAL, and LOX were used as a query against the double haploid Pahang (*Musa acuminata*) banana genome to understand the interacting protein partners. Information regarding interacting partners was obtained based on text mining, experiments, databases, co-expression, neighborhood, gene fusion, and co-occurrence results. Query protein and their interacting protein domain information in addition to the number of interacting partners belonging to a particular domain in the network were analyzed. A degree-sorted network was constructed using transcription factor, defense related genes, and their interacting proteins. Protein domains of all the interacting partners were analyzed to understand the functional relevance and their association with query protein. A medium confidence level of 0.40 and minimum of 10 interactors were used as parameters for the construction of the network.

Analysis of protein-protein interaction network between WRKY33, MAPK, CERK1, PAL, and LOX proteins was obtained using the STRING database. It was exported to Cytoscape 3.9 version for analysis. Using STRING and enrichment

map applications in Cytoscape, STRING enrichment was performed and a map was constructed. Functional enrichment was performed by merging all the five proteins with their interacting partners.

RESULTS

Molecular Confirmation of *Foc* Isolates

Amplification of internal transcribed spacers of eight different isolates of *Foc* for ITS 1 and ITS 4 regions with specific primers yielded an expected amplicon size of 560 bp. Nucleotide sequences of amplicons pertaining to eight different isolates confirmed the identity of *Foc*. The sequences subjected to multiple alignments were submitted to GenBank and were provided with accession numbers, *viz.,* *Foc* KP MW436477, *Foc* KP MW 436476, *Foc* NP1 MW436482, *Foc* NP2 MW436483, *Foc* KP2 MW436485, *Foc* RS2 MW436581, *Foc* RS3 MW436484, and *Foc* RS1 MW43658. Phylogenetic analysis of *Foc* isolates revealed the presence of three major clusters. Cluster 1 comprised *Foc* KP MW 436476, *Foc* KP MW436477, and *Foc* NP1 MW436482. Cluster 2 had five isolates including *Foc* NP2 MW436483, *Foc* KP2 MW436485, *Foc* RS2 MW436581, *Foc* RS3 MW436484, and *Foc* RS1 MW43658. Cluster 3 comprised *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) as an out group (**Supplementary Figure S1**). Based on the pathogenicity study, *Foc* KP was most virulent compared to other isolates. Sequencing of *Six*13c for the isolate *Foc* KP confirmed the presence of race 4 bearing the accession number MW323435. It had similarity with *Foc* isolate BRIP62892 bearing the accession number KX435021 and also matched with VCG group 0122 pertaining to the Cavendish group (AAA) of Philippines origin. Hence, *Foc* KP was used throughout the study.

Antifungal Activity of *B. velezensis* YEBBR6

Bacillus velezensis YEBBR6 inhibited the mycelial growth of *Foc* KP up to 63% compared to untreated control (**Supplementary Figure S2**). Other bacterial endophytes, *B. albus* YEBN2, *A. xylosoxidans* YEBRH2, *Beijerinckia fluminensis* YEBRT4, *Acinetobacter refrigeratoris* YEBFR1, and *B. endophyticus* YEBFL6 were not equally effective as *B. velezensis* YEBBR6 in the suppression of mycelial growth of *Foc* KP *in vitro*.

GC/MS Analysis of VOCs/NVOCs Bioactive Metabolites Extracted From the Zone of Inhibition Produced During the Interaction of *B. velezensis* YEBBR6 With *Foc* KP

Fifteen bioactive metabolites were characterized through GC/MS from the zone of inhibition during the ditrophic interaction between *B. velezenzis* YEBBR6 and *Foc* KP. They included dihydro acridine, nonanol, hexadecanoic acid, oleic acid, clindamycin, 5-hydroxymethylfurfural, azulene, 3 amino-4 hydroxy phenyl sulfone, campholic acid, procyclidine, linoelaedic acid, acetylvaleryl, allobarbital, aminomorpholine, and 3-thiazolidine carboxamide (**Supplementary Figure S3** and **Supplementary Table S1**). Six bioactive metabolites were produced by *B. velezensis* YEBBR6 in the absence of *Foc* KP. They were identified as 1,3-propanediol, clindamycin, 4H-pyran, pentanoic acid, acetaldehyde, and hexadecanoic acid (**Supplementary Figure S4** and **Supplementary Table S2**). Bioactive metabolites produced by *Foc* KP were identified as 2-propen-1-ol, 3H-pyrazol-3-one, hexanoic acid, cyclohexan, 1H-azonine, trioxsalen, and butanamide (**Supplementary Figure S5** and **Supplementary Table S3**). The Venn diagram of differentially expressed bioactive metabolites during the interaction of bacterial endophyte *B. velezensis* YEBBR6, either with *Foc* KP or without *Foc* KP, and *Foc* KP alone revealed that *B. velezensis* YEBBR6 produced six bioactive metabolites. But, during the interaction of *B. velezensis* YEBBR6 with *Foc* KP, 15 bioactive metabolites were produced. Comparison of bioactive metabolites between *B. velezensis* YEBBR6 alone and *B. velezensis* YEBBR6 with *Foc* KP revealed the production of two bioactive metabolites in common, *viz*., hexadecanoic acid and clindamycin (**Figure 1**).

Plant Growth Promotion and Physiology of Micropropagated Banana cv. Karpooravalli (ABB) Plantlets Biohardened With Bacterial Endophytes

Micropropagated banana plantlets treated with *B. velezensis* YEBBR6, *B. albus* YEBN2, *A. xylosoxidans* YEBRH2, *Beijerinckia fluminensis* YEBRT4, *Acinetobacter refrigeratoris YEBFR*1, and *B. endophyticus* YEBFL6 increased plant growth parameters (**Figure 2**). Among the bacterial endophytes, *B. velezensis* YEBBR6 significantly increased pseudostem height (38.63 cm), pseudostem width (5.9 cm), leaf area (1.290 m^2), root length (54.75 cm), and leaf emergence rate (Phyllochron) (6.0), compared to 11.21 cm, 3.2 cm, 0.09 m^2, 36.1 cm, and 12.0 in control plants, respectively (**Figure 3A** and **Supplementary Figures S6–S10**). *B. velezensis* YEBBR6-treated banana plants also enhanced the photosynthetic rate to 24.61 mol m^{-2} s^{-1} against 13.09 mol m^{-2} s^{-1} in non-bacterized control plants. Further, transpiration rate and stomatal conductance in *B. velezensis*-treated banana plants was 3.95 mol.cm^{-2} s^{-1}, 1.01 mmol m^{-2} s, while it was 2.64 mol.cm^{-2} s^{-1}, 0.06 mmol m^{-2} s in non-bacterized control plants. Biohardened banana plants had higher levels of total chlorophyll (1.29 mg/g^1), chlorophyll stability index (88.6%), and relative water content (83.3%) than non-bacterized control plants (**Supplementary Table S4**).

Effect of Bacterial Endophytes on the Suppression of *Fusarium* Wilt of Banana Plantlets

Banana plantlets biohardened with *B. velezensis* YEBBR6, *B. albus* YEBN2, *Achromobacter xylosoxidans* YEBRH2, *Beijerinckia fluminensis* YEBRT4, *Acinetobacter refrigeratoris* YEBFR1, and *B. endophyticus* YEBFL6 against *Foc* KP indicated that banana plantlets biohardened with *B. velezensis* at 10 ml/plant had zero incidence of wilt. But, the banana plantlets biohardened with *B. albus* and *Acinetobacter refrigeratoris* had 10 % wilt against 30 % in banana plants biohardened with *B. endophyticus*. However, 100 % wilt incidence was observed in inoculated control (**Figure 3B** and **Supplementary Table S5**). As *B. velezensis* YEBBR6 was

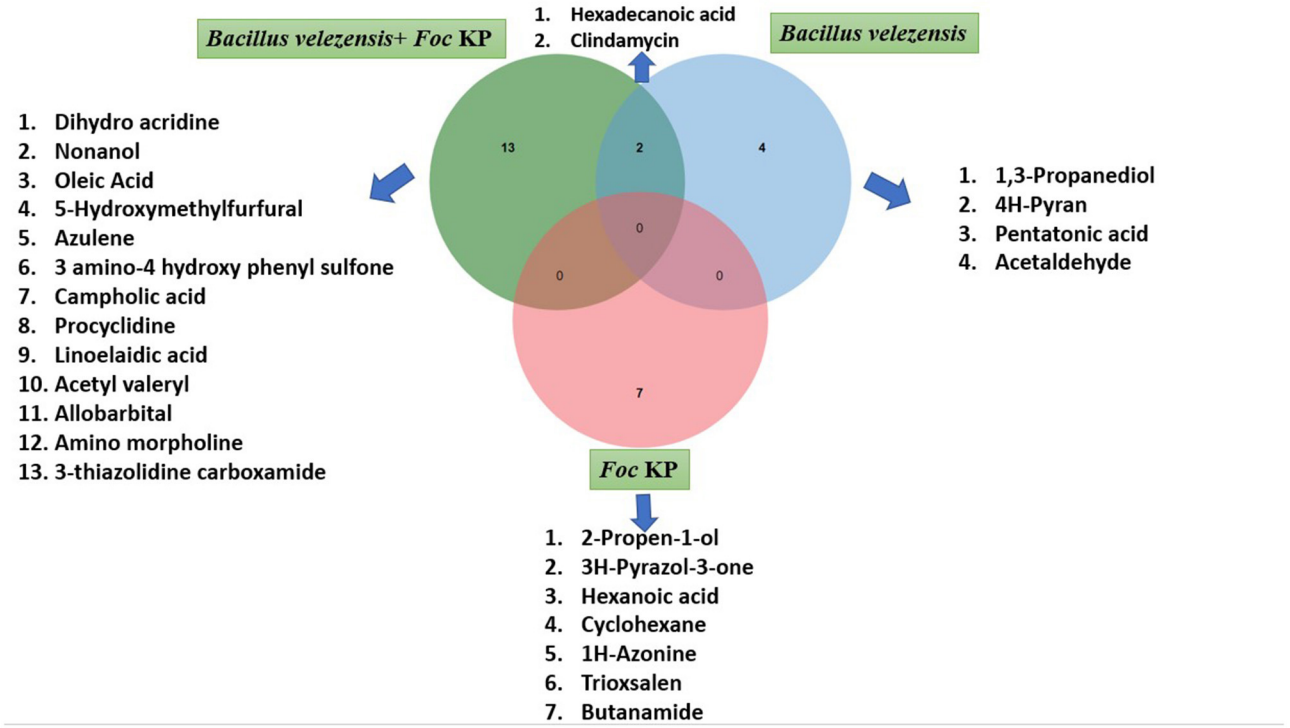


FIGURE 1 | Comparative distribution of VOCs/NVOCs produced by *B. velezensis* YEBBR6, *B. velezensis* YEBBR6 + *Foc* KP, and + *Foc* KP alone.

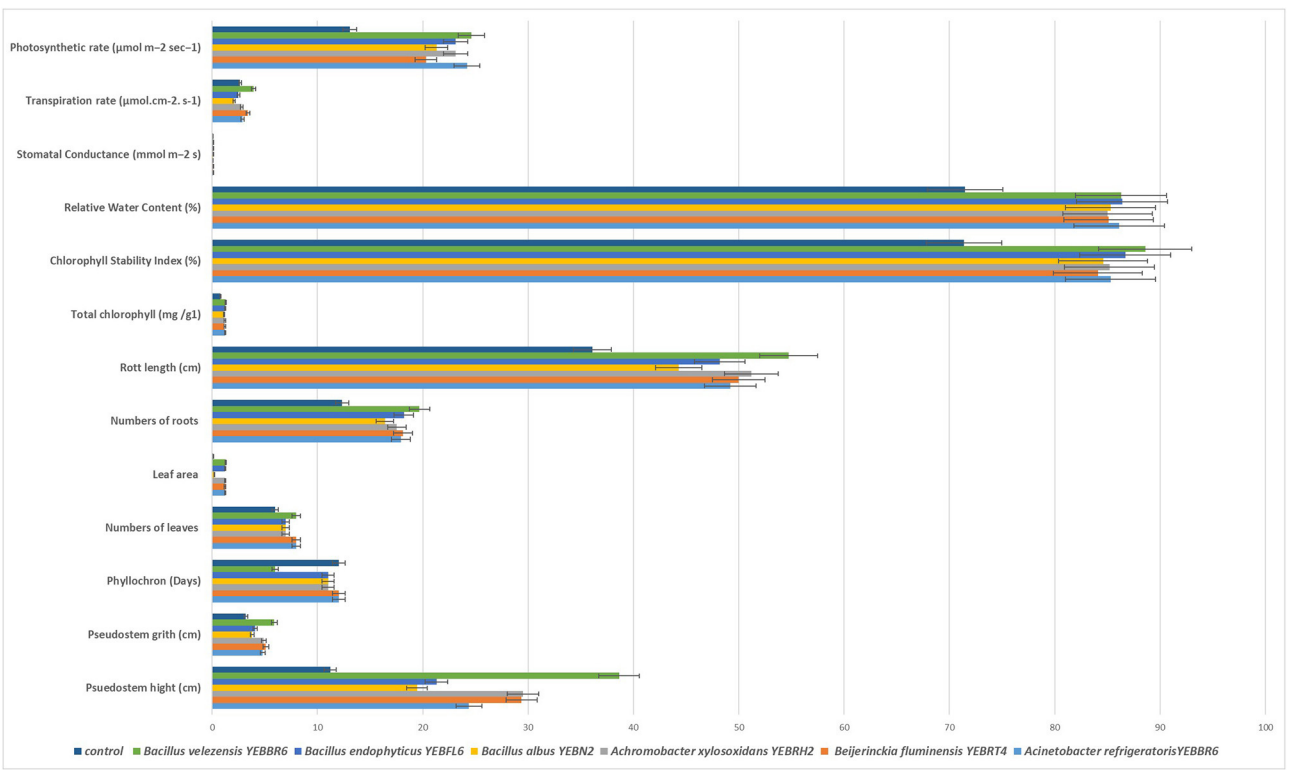


FIGURE 2 | Phenotypic response and plant growth promotion of micropropagated banana plantlets biohardened with bacterial endophytes.

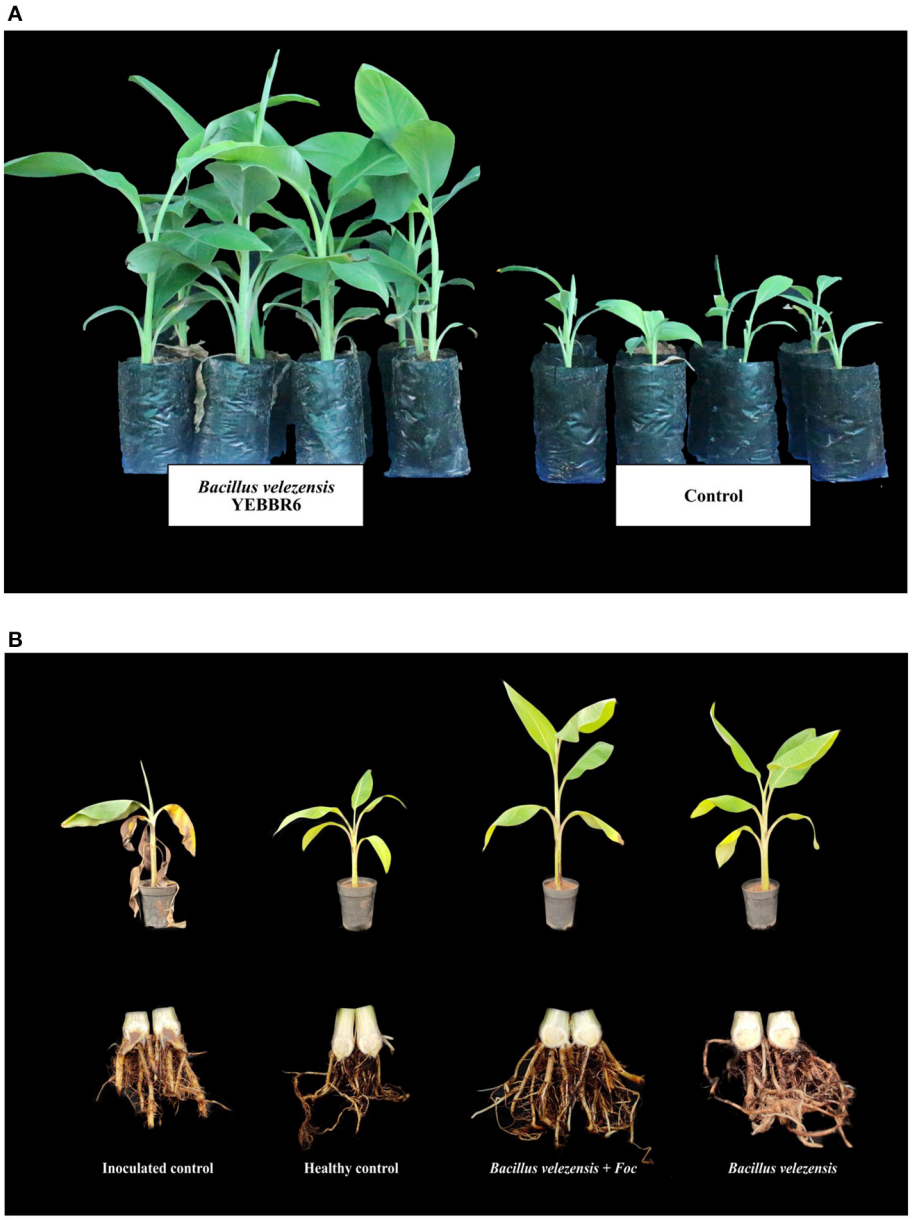


FIGURE 3 | (A) Plant growth promotion of micropropagated banana plantlets biohardened with *Bacillus velezensis* YEBBR6. **(B)** Effect of *Fusarium* wilt on biohardened micropropagated Karpooravalli banana plantlets with *Bacillus velezensis* YEBBR6 against *Foc* in pot culture.

effective in the suppression of *Fusarium* wilt, it was subsequently used for further studies.

Effect of *B. velezensis* YEBBR6 on Root Colonization of Banana Plantlets

Analysis of the roots of banana plantlets biohardened with *B. velezensis* YEBBR6 using scanning electron microscopy confirmed the colonization of bacterial cells on the root surface. Agglomerates of bacterial cells resulted in the formation of biofilm on the root surface (**Figure 4A**). Besides, the roots of banana plantlets biohardened with *B. velezensis* YEBBR6 followed by challenge inoculation with *Foc* KP witnessed hyperparasitism of *Foc* KP mycelium and microconidia by the bacterial cells of *B. velezensis* (**Figures 4B,C**). However, in pathogen-inoculated control, proliferation of microconidia was noticed along the root zone of banana plantlets (**Figure 4D**).

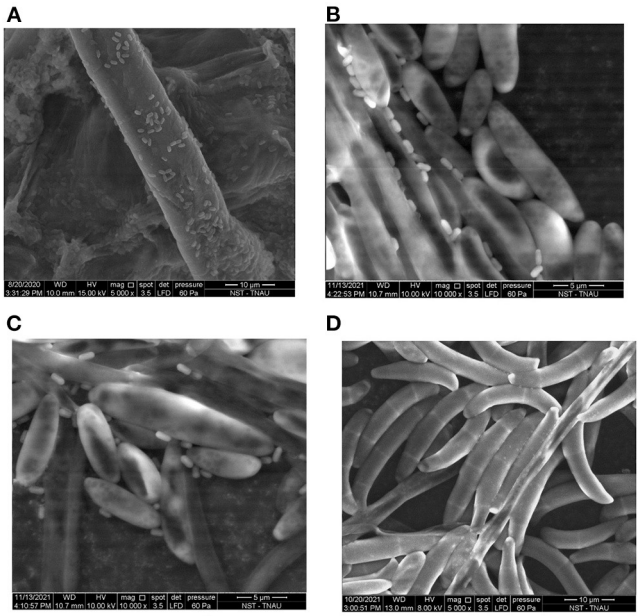


FIGURE 4 | (A) Colonization of *Bacillus velezensis* YEBBR6 in the root zone of biohardened banana plantlets. **(B,C)** Colonization of *Bacillus velezensis* YEBBR6 in the mycelium and microconidia of *Foc* KP on biohardened banana plantlets after challenge inoculation with *Foc* KP. **(D)** Colonization of micro and macroconidia in infected plantlets after challenge inoculation with *Foc* KP.

Induction of Defense Gene Transcripts in Banana Plantlets Biohardened With *B. velezensis* YEBBR6 Challenged With *Foc* KP

Biohardening of banana plantlets with *B. velezensis* YEBBR6 challenged with or without *Foc* KP altered the expression of the WRKY transcription factor, MAPK, chitin elicitor receptor kinase, lipoxygense, and PAL genes responsible for plant defense. Irrespective of different treatments, the WRKY 33 transcript was downregulated in all the treatments at 0 h. The transcription rate of the WRKY 33 gene was affected immediately after inoculation with *Foc* KP and in biohardened plants. The level of the transcript in *Foc* KP-inoculated control increased after 24 h and declined after 48 and 72 h. However, the transcript of WRKY 33 was upregulated in banana plantlets biohardened with *B. velezensis* at 24, 48, and 72 h after treatment. Interestingly, a 1.87-fold increase of the WRKY 33 gene transcript was observed in banana plantlets biohardened with *B. velezensis* YEBBR6 challenged with *Foc* KP. But, in untreated healthy control, upregulation of WRKY 33 (0.33-fold) was noticed only at 72 h (**Figure 5A**).

The expression level of MAPK transcripts varied between different treatments. Banana plantlets biohardened with *B. velezensis* YEBBR6 challenged with *Foc* KP increased the expression of MAPK transcripts up to 2.32-fold after 72 h in comparison with biohardened plants that were not challenged with *Foc* KP. Besides, only a 0.69-fold change of the WRKY 33 transcript was noticed in pathogen-inoculated control after 48 h and it decreased further after 72 h of inoculation (**Figure 5B**).

Induction of the chitin elicitor receptor kinase (CERK 1) transcript associated with innate immune response was initiated after 24 h of inoculation with pathogen *Foc* KP. However, upregulation was more pronounced after 72 h in plantlets biohardened with *B. velezensis* YEBBR6 coupled with challenge inoculation of *Foc* KP. The expression of the CERK1 transcript was 2.2-fold higher than in biohardened plants that had not been inoculated with *Foc* KP. Furthermore, the expression level of CERK1 transcripts was reduced in untreated healthy control compared to pathogen-inoculated and biohardened plantlets challenged with *Foc* KP (**Figure 5C**).

Lipoxygenase (LOX) is a key enzyme involved in the induction of the immune response in plants, therefore attempts were made to understand the ability of *B. velezensis* YEBBR6 to modulate immune response against *Foc* KP. Biohardened plants challenged with *Foc* KP increased the transcript level of fatty acid dioxygenase LOX up to 2.6-fold after 72 h of inoculation. The expression of the LOX transcript was 1.6-fold greater in *B. velezensis* YEBBR6 and was downregulated after 72 h. Comparison on the expression of the LOX transcript in inoculated control reflected a 1.7-fold change after 72 h, while in healthy control, there was no increase in the LOX gene transcripts (**Figure 5D**).

Assessing the expression of phenylalanine ammonia lyase (PAL) revealed a significant increase of PAL transcripts in *B. velezensis* YEBBR6 biohardened banana plantlets challenged with *Foc* KP. After 48 h of challenge inoculation with *Foc* KP, the level of induction of the PAL transcript was 1.9 times higher than other treatments. After 48 h, the PAL transcript in *Foc* KP-inoculated control was observed only up to 0.6-fold, and decreased after 72 h. However, the activity of PAL was downregulated in healthy control (**Figure 5E**).

Analysis of Protein-Protein Interaction Between Defense Genes in *B. velezensis* YEBBR6 Biohardened Banana Plantlets Challenged With *Foc* KP

STRING analysis was performed to understand the functional association of defense-related genes with other proteins and their conserved domains. From our analysis, most of the interacting proteins for each query protein had similar conserved domains and were clustered together in the network. Protein sequences of defense-related genes were used as input to retrieve the interacting partners based on the double haploid Pahang (*Musa acuminata*) banana genome. Details on the query protein and their interacting protein domain and number of interacting partners in the network are listed in **Supplementary Table S6**. The degree-sorted network constructed using transcription factor and defense-related genes and their interacting proteins are illustrated in **Figure 6**.

Protein-protein interaction of WRKY transcription factor indicated the involvement of a large protein family with diverse functions. They were expressed in response to pathogens, elicitors, and defense-related phytohormones such as salicylic

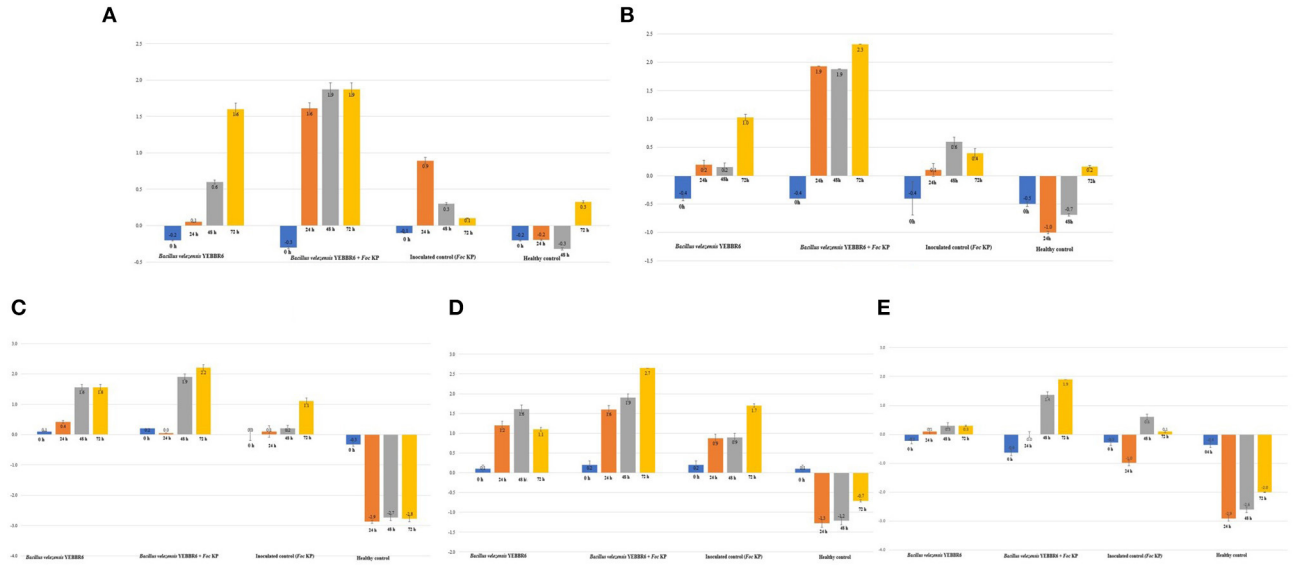


FIGURE 5 | (A) Expression pattern of WRKY 33 transcription factor in biohardened banana cv. Karpooravalli (ABB) under mono, di, and tritrophic interactions at different time periods. **(B)** Expression pattern of MAPK in biohardened banana cv. Karpooravalli (ABB) under mono, di, and tritrophic interactions at different time periods. **(C)** Expression pattern of CERK 1 in biohardened banana cv. Karpooravalli (ABB) under mono, di, and tritrophic interactions at different time periods. **(D)** Expression pattern of LOX in biohardened banana cv. Karpooravalli (ABB) under mono, di, and tritrophic interactions at different time periods. **(E)** Expression pattern of PAL in biohardened banana cv. Karpooravalli (ABB) banana under mono, di, and tritrophic interactions at different time periods.

acid (SA) or jasmonic acid (JA). Protein-protein interaction of MAPK revealed that it belongs to the kinase family and interacted with G protein domains and protein tyrosine phosphatase domains similar to chitin elicitor receptor kinase 1-like protein. Besides, functional domains of five out of ten interacting proteins were uncharacterized in the given network. CERK 1 protein pertaining to the protein kinase family protected plants at multiple layers against invading pathogens by conferring PAMP-triggered immunity (PTI) or effector-triggered immunity (ETI). Regardless, CERK proteins interacted with the LysM domain and guanine nucleotide-binding domain (**Supplementary Table S6**). Protein interaction of LOX indicated that it interacted with proteins containing LOX, phospholipase, and PLAT/LH2 conserved domains. These proteins were associated with membrane or lipid-associated proteins. Similarly, protein-protein interaction of PAL reflected that chalcone synthase (CHS) was essential for the formation of 4,2′, 4′,6′-tetrahydroxychalcone, responsible for the biosynthesis of anthocyanin pigments, anti-microbial phytoalexins, and flavonoid inducers of *Rhizobium* nodulation genes. Nonetheless, stilbene synthases (STSs) occurred in a limited number of unrelated plants and synthesized the backbone of stilbene phytoalexins that have antifungal properties and contribute to pathogen defense. Despite the other genes, PAL also induced defense against pathogens by triggering other enzymes linked with different pathways.

Merging of WRKY33, MAPK, CERK1, PAL, and LOX resulted in the formation of an enlarged network comprising of 52 nodes. Three isoforms of guanine nucleotide-binding proteins interacted commonly with CERK and MAPK protein targets. Apart from this, there was no common interacting proteins between the five protein targets. Functional enrichment analysis resulted in the formation of more than five clusters. A heat cluster map was generated based on the functional relevance of each of the proteins. In the enrichment figure, each cluster was linked with the query protein to indicate the commonality and the difference observed with the five query proteins and their interaction partners. Based on the enrichment map, domain, and pathway information of five proteins, CERK1 was linked with the LysM domain and guanine nucleotide-binding protein domain. LOX was linked with linoleic acid, alpha-linolenic acid, arachidonic acid metabolism, ether lipid metabolism, glycerophospholipid metabolism, lipoxygenase, phospholipase, and PLAT/LH2 conserved domains. The MAPK gene was associated with G protein domains and protein tyrosine phosphatase domains. While, PAL was coordinated with AMP binding, chalcone synthase, polyketide synthase, and thiolase-like domains. The WRKY transcription factor was associated with the VQ5 domain and WD40 repeat containing domain (**Figure 7**).

DISCUSSION

Fusarium wilt of banana, caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*), is one of the most devastating diseases hindering the banana industry. A recent outbreak of *Foc* tropical race 4 (*Foc* TR4) can infect and destroy almost all banana cultivars. Hence, the present study, using molecular approaches to understand the versatile nature and distribution of *Foc* races infecting banana in Tamil Nadu province of South India through ITS 1 and 4 and Six 13c, revealed the presence of race 4 (*Foc* KP–MW323435).

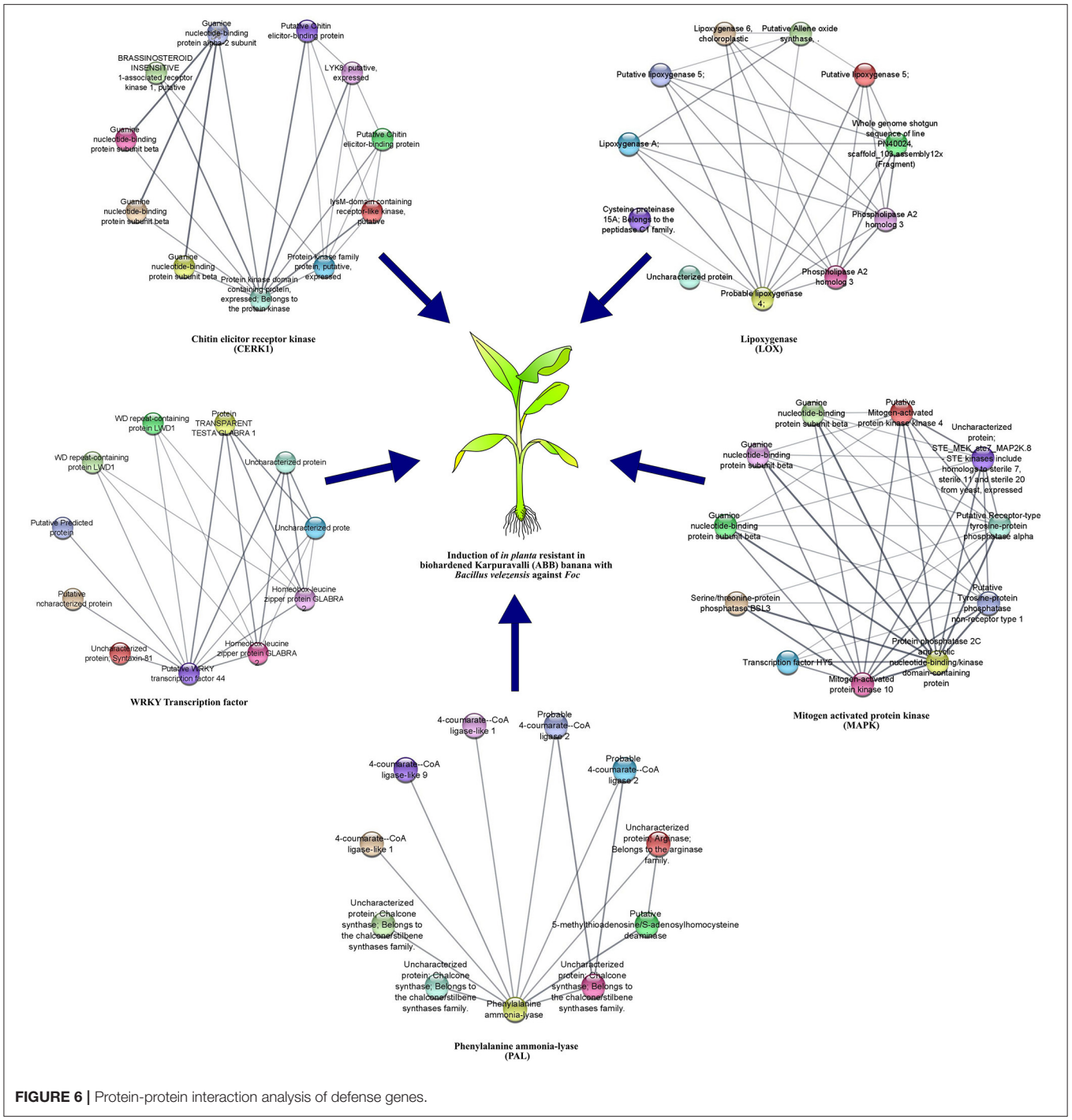


FIGURE 6 | Protein-protein interaction analysis of defense genes.

Likewise, ITS region has been used as a molecular marker to confirm the identity of *Foc*-infecting *Musa* spp. (ABB) in Southern Mexico (Leong et al., 2009; Maldonado-Bonilla et al., 2019). Further, in the present study, sequencing of Six 13c-343 for the isolate *Foc* KP confirmed the presence of race 4 bearing the accession number MW323435. Sequences of the isolate *Foc* KP had the similarity with *Foc* isolate BRIP62892 bearing the accession number KX435021. Further, it also matched with VCG group 0122 pertaining to the Cavendish (*Musa* AAA) group of banana with Philippines origin (Czislowski et al., 2018; Carvalhais et al., 2019). Similarly, Wong et al. (2019) confirmed the presence of *Foc* TR4 in Peninsular Malaysia based on transcription elongation factor (TEF-1) and reported the shift in genetic variability among *Foc* isolates. Despite the existence of different races, management of race 4 remains a challenging task. Though fungicides are effective, usages of

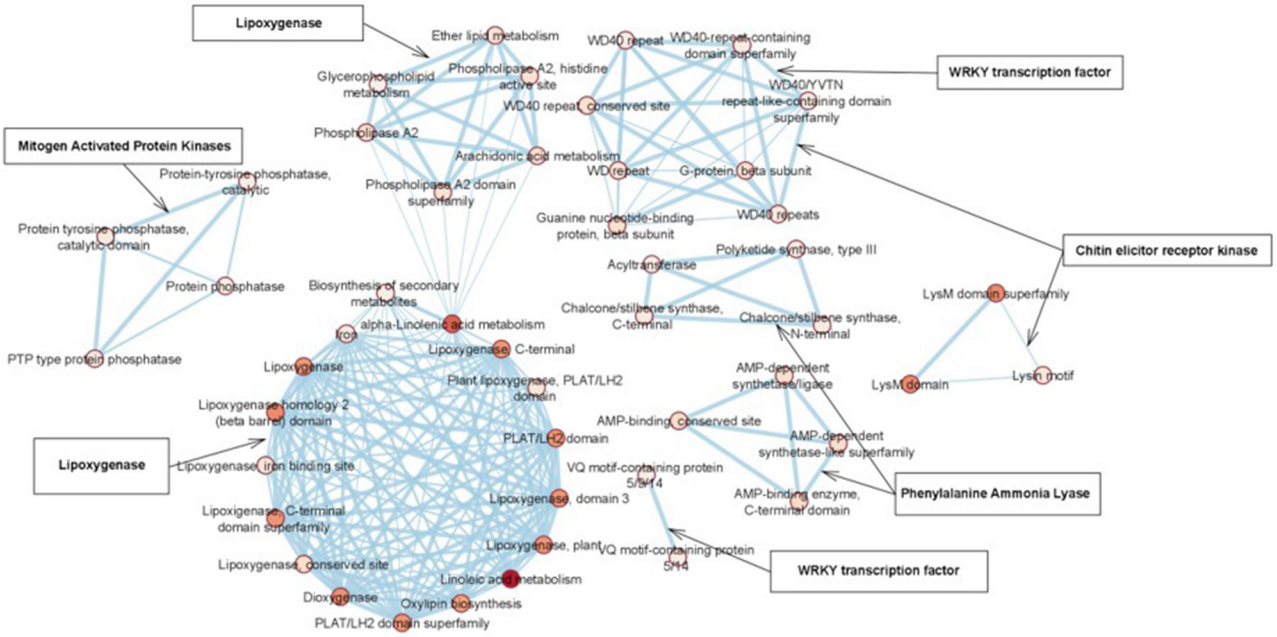


FIGURE 7 | Functional enrichment analysis of defense genes in biohardened Karpooravalli (ABB) banana plantlets.

fungicides are discouraged due to environmental concern and resistance development among plant pathogens (Raza et al., 2017). Moreover, none of the fungicides confer 100 % protection against *Foc*. Due to the detrimental effect of chemical fungicides on the environment, human health, and other living organisms, researchers have focused on discovering potential biocontrol candidates as a viable alternative for plant disease management (Syed Ab Rahman et al., 2018).

Considering this critical situation, structuring the rhizosphere and endosphere with potential bacterial antagonists in the susceptible banana cultivars will pave the way for the management of *Foc*. Among all bacterial endophytes, the *Bacillus* species were exploited largely for suppression of soil-borne pathogens around the globe due to rapid growth and the ability to synthesize a large number of secondary metabolites which play a key role in antibiosis against deleterious microorganisms (Radhakrishnan et al., 2017; Fira et al., 2018; Aloo et al., 2019). Besides, in the recent past, biological control of plant diseases has gained popularity as an eco-friendly disease management strategy.

Based on the significance in the management of *Fusarium* wilt of banana, our finding has furthered the introduction of a potential endophyte *B. velezensis* YEBBR6 with multifaceted attributes contributing to the suppression of *Foc* KP. Similarly, the versatile nature of antifungal secondary metabolites from endophytic *Brachybacterium paraconglomeratum* isolated from the resistant cultivar YKM5 was also responsible for the suppression of *Foc* under *in vitro* conditions (Saravanan et al., 2021a). The VOCs and NVOCs are also referred to as small signaling molecules (SSMs), which are involved in cellular crosstalk. They play a significant role in competition, synergistic interaction, and communication (Adnani et al., 2017). SSMs from the antagonistic microflora also promote the growth of symbionts and inhibit plant pathogens (Khalid and Keller, 2021). Like the cellular crosstalk mediated by small signaling molecules between the beneficial microbiome and inimical microbes dwelling in the rhizosphere, small signaling molecules were also induced during the interaction between the fungal pathogen and bacterial antagonist *in vitro*. Likewise, co-culturing of *B. velezensis* YRBBR6 along with *Foc* KP also induced the secretion of secondary metabolites, *viz*., dihydroacridine, nonanol, hexadecanoic acid, oleic acid, clindamycin, 5-hydroxymethylfurfural, azulene, 3 amino-4 hydroxy phenyl sulfone, campholic acid, procyclidine, linoelaedic acid, acetylvaleryl, allobarbital, aminomorpholine, and 3-thiazolidine carboxamide. Gatasheh et al. (2017) reported that dihydroacridine disrupted DNA synthesis and served as a DNA intercalating agent in several microorganisms leading to broad-spectrum antimicrobial activity. Mohamad et al. (2018) explained that *B. atrophaeus* strain XEGI50 inhibited the mycelial growth of *V. dahliae* by producing 13 putative compounds, including 1,2-benzenedicarboxylic acid, bis (2-methylpropyl) ester 9,12-octadecadienoic acid (Z, Z)-, methyl ester 9-octadecenoic acid, methyl ester decanedioic acid, bis(2-ethylhexyl) ester, and hexadecanoic acid. Walters et al. (2004) reported that fatty acids, *viz.,* linolenic acid, linoleic acid, erucic acid, and oleic acid promoted plant growth and possessed antifungal action against *Rhizoctonia solani, Pythium ultimum,* and *Pyrenophora avenae*. The antifungal activity of nonanol produced by endophytic *B. velezensis* ZSY-1

was also reported by Gao et al. (2017) against *A. solani* and *B. cinerea*. Zhang et al. (2017) reported the broad spectrum antimicrobial activity of a-phellandrene and nonanal against Penicillium cyclopium. Further, Guay (2007) reported the antimicrobial action of clindamycin against different microbes. Abd Alhameed et al. (2020) reported the antimicrobial activity of thiazolidine-2,4-dione carboxamide against bacteria and fungus. To our surprise, *B. velezensis* YRBBR6 also produced dihydroacridine, clindamycin, and nonanol bestowed with antifungal action against *Foc* KP. Thus, the bacterial endophyte *B. velezensis* YRBBR6 is unique in the sense that it produced novel biomolecules responsible for the suppression of mycelial growth of *Foc* KP, which has not been reported earlier by other researchers for the management of *Foc*. Hence, it is hypothesized that an array of secondary metabolites produced by *B. velezensis* YRBBR6 might be responsible for the suppression of *Foc* KP.

Endophytic *B. velezensis* YRBBR6 not only possessed antifungal property but also promoted plant growth. This was also endorsed by the research findings of Compant et al. (2016) who reported that the bacterial endophytes improved plant health and growth through a variety of mechanisms, including phytohormone synthesis, nitrogen fixation, phosphate solubilization, stimulation of defense responses, and reduction of abiotic stress by lowering ethylene levels. Yuan et al. (2013) reported that *B. amyloliquefaciens* strain NJN-6 served as a biofertilizer and promoted the growth of micropropagated banana plants treated with *B. amyloliquefaciens* NJN-6. Biohardening of banana cv. Karpooravalli (ABB) plants with *B. velezensis* (YEBR6) promoted plant growth through the increase in pseudostem height, width, leaf area, root length, and emergence of new leaves. The research finding of Gamez and his associates emphasized that the banana plants inoculated with rhizobacteria increased plant height, leaf number, leaf area, pseudostem thickness, root and shoot fresh weight, and root and shoot dry weight (Gamez et al., 2019). Micropropagated banana plantlets treated with *Bacillus* and *Pseudomonas* promoted plant growth by increasing pseudostem height, width, number of leaves, leaf area, and yield parameters (Kavino et al., 2011). Kavino et al. (2014) also observed a significant difference in phyllochrons in biohardened banana plants compared to untreated control. Further, Ajit kumar et al. (2020) also reported that bacterial endophytes improved pseudostem height, pseudostem width (diameter), number of roots, and total number of leaves in banana plants. Rajamanickam et al. (2018) recorded increased growth parameters in biohardened banana plants compared to untreated control. Thus, the phytobiome plays a key role in improving plant health. Colonization of the endosphere and rhizosphere by bacterial endophytes enhanced plant growth by mobilizing nitrogen, production of phytohormones, acquisition of nutrients, and also conferred resistance to biotic and abiotic stresses (Kandel et al., 2017). Thus, to harness the potential benefits mediated through endophytes, colonization of the rhizosphere and endosphere is very crucial. Successful colonization of the rhizoplane by bacterial antagonists is a prerequisite for biocontrol and plant growth promotion (Gao et al., 2016; Kang et al., 2019). Considering the significance of colonization by endophytes, the present investigation confirmed the colonization of endophytic *B. velezensis* YEBBR6 on the rhizosphere. Similarly, scanning electron microscopy analysis of a cucumber root surface applied with *B. amyloliquefaciens* UCMB5113 revealed the colonization of bacterial cells and formation of biofilm leading to plant growth promotion (Palmqvist et al., 2015). In corroboration with our findings, micropropagated banana plantlets inoculated with *B. velezensis* RFP-N67 colonized roots xylem cells in a successful manner. On the other hand, it also entered inside the root at a higher population density of bacterial cells. Likewise, we could observe the colonization of the banana pseudostem by endophytic isolate YEBBR6 through SEM. It was also speculated that *B. velezensis* RFP-N67 proliferated normally in banana to impose their biocontrol functions, and in particular during the presence of *Foc*, antagonistic bacterial isolate RFP-N67 can quickly inhibit the growth of the pathogen (He et al., 2021). Similar responses were also induced by the antagonistic bacterial endophyte in the presence of *Foc* KP responsible for quenching the infection of the wilt pathogen. It was also witnessed through the hyperparasitic behavior of the antagonistic bacteria *B. velezensis* YEBBR6 in the micropropagated banana cv. Karpooravalli (ABB) plantlets through scanning electron micrographs.

As *B. velezensis* YEBBR6 promoted plant growth, we further focused our studies to assess the impact of the bacterial antagonist on physiological attributes of banana plantlets. Physiological attributes contribute toward plant defense and serve as the medium of interaction with the environment and climatic conditions. These attributes are linked with the physiological development associated with cellular processes including transpiration, photosynthesis, stomatal conductance, relative water content, and regulation of plant hormones (Shah et al., 2020). In our study, micropropagated banana plants treated with *B. velezensis* YEBBR6 increased major physiological parameters including photosynthetic rate, stomatal conductance, relative water content, and chlorophyll stability index compared to untreated control. Similarly, Shamsuddin et al. (2000) reported that inoculation of banana plantlets with PGPR had the highest photosynthetic rate and stomatal conductance. Besides, it also increased stomatal conductance and lowered proline concentration in leaves of banana seedling grown under abiotic stress (Shamsuddin et al., 1999). Similarly, biohardened banana plants with *B. velezensis* YEBBR6 also increased stomatal conductance.

Soil application of *B. amyloliquefaciens* NJN6 to banana plantlets increased the population density of beneficial rhizomicrobiome and thus aided in the management of *Fusarium* wilt by decreasing colonization of *Foc* in the banana rhizosphere (Xue et al., 2015). *In vitro* bacterization of tissue-cultured banana plantlets with endophytic *B. subtilis* EPB56 and EPB10 reduced *Fusarium* wilt by 78 % compared to pathogen-inoculated control (Kavino and Manoranjitham, 2018). *B. velezensis* isolates (Y6 and F7) enhanced the antagonistic activity against banana *Fusarium* wilt (Cao et al., 2018). Combined application of *B. velezensis* H-6 with acid soil ameliorant (ASA) suppressed the incidence of *Foc* race 4 up to 63.3% to 66.7%. Besides, it also enhanced growth promotion in banana plants (Huang et al., 2019). Furthermore, Wang et al. (2013) reported

that *B. amyloliquefaciens* W19 acted in synergy with organic fertilizer to reduce the incidence of *Fusarium* wilt other than plant growth promotion. Hitherto, biohardening of banana cv. Karpooravalli (ABB) with endophytic *B. velezensis* YEBBR6 completely protected the plantlets from the establishment of the host pathogen relationship of *Foc* KP compared to the other bacterial endophytes investigated in the present study. Bacterial endophyte *B. velezensis* YEBBR6 not only suppressed the host pathogen relationship of *Foc* KP, but also reduced mycelial proliferation *in vitro* and promoted plant growth. Hence, attempts were also made to understand the regulation of transcription factors and defense genes expressed during mono, di, and tritrophic interactions with *B. velezensis* YEBBR6 and *Foc* KP in biohardened banana plantlets. Bacterial endophytes can induce immune response in plants by its macromolecules and MAMP molecules via host signals (Wei et al., 1991). Profiling the expression in biohardened micropropagated banana cv. Karpooravalli (ABB) with *B. velezensis* YEBBR6 had a clear upregulation pattern of WRKY 33 transcription factor, PAL, LOX, MAPK, and CERK 1 in biohardened plants challenged with *Foc* KP compared to inoculated control and untreated control plants. Transcription parameters regulate a broad range of signal transduction pathways with various tasks, and thus play a key role in the induction of plant defense. WRKY, being a group of transcription regulators in plants, can bind to box in promoters of target genes to regulate transcription (Eulgem et al., 2000). WRKY transcription factors also coordinate a variety of signaling pathways and have a crucial regulatory role in plant defense responses (Zhang et al., 2019). They also regulate pathogen-associated molecular pattern-triggered immunity (PTI) and effector-triggered immunity (Chen et al., 2019).

WRKYs also interact with mitogen-activated protein kinase (Rushton et al., 2010; Mao et al., 2011), MAP kinase kinase kinase (MEKK) (Guan et al., 2014), calmodulin (Rushton et al., 2010), and histone deacetylases (HDAs). Zhang et al. (2019) recorded the expression of seven different WRKY genes, including WRKY4 (Ma10_g03630), WRKY22 (Ma10_g06870), WRKY25 (Ma06_g34370), and WRKY26 (Ma03_g09270, Ma06_g01150, Ma08_g01650, and Ma11_g18140) during *Foc* infection and suggested that expression of these WRKY genes might be responsible for the constitutive defense mechanism. Thus, the multiple fold increase of WRKY 33 transcription factor in bacterized banana cv. Karpooravalli (ABB) plants by *B. velezensis* YEBBR6 might have triggered the constitutive defense response against *Foc* KP. In agreement with our finding, Vanthana et al. (2019) reported that MAMP molecules of *B. velezensis* VB7 increased the expression of the WRKY gene in tomato plants compared to control against GBNV. Next to the WRKY transcription factor, phosphorylation of appropriate protein substrates is highly essential to catalyze the expression of defense genes and regulation of cell functions in the midst of biotic and abiotic stress through the association of MAPKs, one of the largest group of transferases (Onyilo et al., 2017; Xu et al., 2017; Jagodzik et al., 2018; Vanthana et al., 2019). In addition, MAPK cascades play an important role in signal transduction and regulate crosstalk between important hormonal pathways including auxin (AUX), abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), ethylene (ET), brassinosteroids (BR), and gibberellins (GA) (Mishra et al., 2006; Rodriguez et al., 2010; Lu et al., 2015). Furthermore, cascades of MAPK are also involved in regulation of signaling related to multiple defense responses, defense hormones, reactive oxygen species (ROS) generation, stomatal closure, defense gene activation, phytoalexin biosynthesis, cell wall strengthening, and hypersensitive response (HR) cell death (Meng and Zhang, 2013). Considering the significant impact of MAPK, our focus on understanding the regulation of MAPK in *B. velezensis* YEBBR6-treated banana plants challenged with *Foc* KP confirmed a 2.42-fold increased expression of the MAPK gene. McNeece et al. (2019) also reported the co-expression of five different types of defense genes related to MAPK influencing PTI and ETI against plant pathogenic *Fusarium* species.

Despite the induction of WRKY 33 and MAPK defense genes, PAL, LOX, and CERK 1 transcripts were also increased in *B. velezensis*-treated banana cv. Karpooravalli (ABB) plantlets compared to in untreated control. PAL, being the first enzyme involved in the phenylpropanoid pathway during biotic and abiotic stress, catalyzes the first step in the phenylpropanoid pathway and regulates defense signaling (Lyne et al., 1976). It is also involved in the conversion of phenylalanine to trans-cinnamic acid which is the entry step for channeling carbon from primary metabolism into phenylpropanoid secondary metabolism in plants (Campbell and Ellis, 1992; Ritter and Schulz, 2004). All these pathways bestowed the metabolites with antifungal activity. Apart from the induction of metabolites with antifungal activity, PAL interacts with chalcone synthase, polyketide synthase, and stilbene synthases which are responsible for antifungal action (Schanz et al., 1992; Okada et al., 2004; Zhu et al., 2004). Induction of PAL in banana plants suppressed *Foc* infection (Wang et al., 2016). Application of bacterial endophytes against *Foc* accumulated defense-related enzymes such as PO, PPO, and PAL (Ajit kumar et al., 2020). Thus, based on protein-protein interaction, increase in the expression of PAL transcripts in banana plantlets biohardened with *B. velezensis* YEBBR6 against *Foc* KP might have simultaneously induced various pathways and proteins responsible for the suppression of *Fusarium* wilt via the induction of SAR. Interestingly, banana plantlets of banana cv. Karpooravalli (ABB) biohardened with *B. velezensis* YEBBR6 against *Foc* KP also increased the transcript levels of CERK 1 and LOX genes. CERK 1, being a cell surface receptor, plays a pivotal role in the induction of innate immunity against biotic and abiotic stresses (Shinya et al., 2014). Expression of CERK 1 also can co-express LysM domain-based defense genes contributing to the immune response against *Foc* KP. Besides, CERK 1 protein and its interacting partners recognize pathogen entry and mediate signaling events leading to suppression of the pathogen. Induction of LOX in biohardened banana cv. Karpooravalli (ABB) plantlets by *B. velezensis* and *Foc* KP might have promoted plant growth and plant defense. Hydroperoxidation products of the LOX pathway are responsible for seed germination, plant growth, development, plant senescence, and plant defense against insect and disease attacks.

Ultimately, the present investigation emphasized that biohardening of micropropagated banana cv. Karpooravalli (ABB) with *B. velezensis* YEBBR6 promoted plant growth and suppressed the infection of *Fusarium* wilt through the induction of WRKY 33 transcription factor, MAPK, and defense genes including PAL, LOX, and CERK 1. Further protein-protein interaction also confirmed the co-expression of different domains involved in innate immunity and growth promotion. Subsequently, *B. velezensis* YEBBR6 also produced antifungal metabolites clindamycin and nonanol which are responsible for inhibiting the mycelial growth of *Foc* KP. Thus, biohardening with multifaceted *B. velezensis* YRBBR6 can be explored for the management of *Fusarium* wilt of banana.

CONCLUSION

Investigation on biohardening of the micropropagated susceptible cultivar cv. Karpooravalli (Pisang Awak ABB) with bacterial endophyte *B. velezensis* YEBBR6 derived from the resistant genotype YKM5 on reprogramming of innate immunity against *Foc* KP revealed the versatile production of metabolically active biomolecules contributing to the suppression of *Foc* KP. On the other hand, biohardening enhanced the growth promotion of banana plantlets by increasing the plant height and production of number of leaves in comparison with untreated control. Scanning electron micrographs also confirmed the colonization of the rhizoplane by *B. velezensis* YEBBR6, followed by hyperparasitism of *Foc* KP in the rhizosphere. Challenge inoculation of biohardened cv. Karpooravalli (Pisang Awak ABB) with *Foc* KP enhanced the transcript level of WRKY transcription factor, MAPK, and other defense genes including CERK 1, LOX, and PAL. Functional enrichment of different defense genes and transcription factors was linked with different domains responsible for growth promotion, induction of systemic resistance, and systemic-acquired resistance. As a result, the current study has opened up the scope for exploring the immense potential of *B. velezensis* YEBBR6 to bioharden micropropagated banana plantlets on a commercial scale to create preimmunized seedlings for the management of *Foc* KP (race 4).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

SN conceptualized the research and was associated with technically guiding and executing the research. RS performed lab experiments. NS and VR carried out the bioinformatics analysis. MK and SV coordinated the experiments associated with defense gene expression. MR, AK, SH, and VM edited the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2022.845512/full#supplementary-material

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