Eur J Plant Pathol
https://doi.org/10.1007/s10658-026-03252-z

Pheno-molecular insights into the biocontrol of banana fusarium wilt by *Trichoderma yunnanense* SB8

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Received: 5 January 2026 / Accepted: 21 May 2026

Abstract Microbial biocontrol agents, particularly *Trichoderma* spp. are increasingly utilized in agricultural production to suppress plant pathogens and reduce reliance on agrochemicals. *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 (FocTR4) is a devastating pathogen causing Fusarium wilt in banana, with virulence largely driven by Secreted in Xylem (SIX) effector proteins that disrupt host defence mechanisms. This study evaluated the biocontrol potential of *Trichoderma yunnanense* strain SB8 against FocTR4 and its influence on the expression of virulence-associated *SIX* genes. Antagonistic assays were conducted followed by in-planta experiment in which banana plants were pre-treated with SB8 prior to FocTR4 inoculation. Disease severity was assessed, and expression levels of selected *SIX* genes (*SIX1i, SIX6, SIX8a, and SIX9a*) were analysed. The results demonstrated that SB8 inhibited FocTR4 growth by 87.21%. Pre-treated banana plants exhibited reduced disease symptoms and severity compared to untreated FocTR4-inoculated controls. Gene expression analysis revealed lower expression levels of *SIX* genes (0.8-fold–2.0-fold change) in banana plants pre-treated with SB8 compared to the higher levels in untreated FocTR4-inoculated plants (1.4-fold–4.2-fold change). Hence, these findings highlight the potential of *T. yunnanense* SB8 as an effective biocontrol agent capable of suppressing FocTR4, thereby promoting sustainable strategy for managing Fusarium wilt in banana cultivation.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10658-026-03252-z.

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Published online: 04 June 2026

Keywords Banana · Fusarium wilt · *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 · *Trichoderma yunnanense* · *Secreted in Xylem*

Introduction

Fusarium wilt, caused by *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 (FocTR4), is one of the most devastating diseases affecting banana production globally (Adhikary et al., 2024; Martinez et al., 2023; Munhoz et al., 2024). FocTR4 invades banana roots, block vascular tissues and impedes the transport of essential minerals within the plant (Swarupa et al., 2014). The pathogen also secretes small effector proteins known as Secreted in Xylem (SIX) into the plant's xylem sap which disrupt the host defence mechanisms and facilitate disease development (Kaliapan et al., 2024).

A major challenge in managing Fusarium wilt is the ability of FocTR4 to survive in the soil in the absence of its host, which undermines agronomic practices such as crop rotation (Raman et al., 2021). Chemical control measures, including corm injection and soil drenching with carbendazim fungicide, have proven to be unsustainable and raise environmental concerns (Cannon et al., 2022). On the other hand, approaches such as soil fumigation, field sanitation, and flood fallowing offer limited control, particularly over large cultivation areas (Damodaran et al., 2020).

As an alternative to chemical fungicides, biological control (biocontrol) using living organisms, known as biological control agents (BCAs) is highly recommended (Collinge et al., 2022) for managing pests and pathogens (Boro et al., 2022; Dwisandi et al., 2024). *Trichoderma* spp., a genus of fungi, is a well-known BCA that promotes plant health and protects against various plant pathogens, including *Fusarium* (Awal et al., 2024; Martinez et al., 2023). They function as biofungicides in agriculture, reducing crop losses through mechanisms such as antibiosis, mycoparasitism, antagonism and induced resistance (Debnath et al., 2020; Prismantoro et al., 2024).

Previous studies have shown that inoculation with *Trichoderma* spp. upregulates genes associated with plant physiological enhancement and abiotic stress tolerance, resulting in improved yields in crops such as rice (Doni et al., 2017), cacao (Bae et al., 2009), tomato (Chacón et al., 2007), and cucumber (Samolski et al., 2012). Specifically, Damodaran et al. (2020), reported that *T. reesei* significantly reduced the disease severity of Fusarium wilt caused by FocTR4, indicating a lower infection rate. Similarly, Wibowo et al. (2013) demonstrated the effectiveness of *T. harzianum* in controlling Fusarium wilt of banana under both *in vitro* and *in vivo* conditions. However, to the best of our knowledge, no study has explored the relationship between SIX gene expression and *Trichoderma* spp. during banana-*FocTR4-Trichoderma* interaction.

This study aims to investigate the potential of *T. yunnanense* SB8, previously shown to promote growth and physiological characteristics of rice plants (Akbari et al., 2024) in antagonizing FocTR4 in *Musa acuminata* cv. 'Berangan'. The expression of *SIX* genes in cv. 'Berangan' plants pre-treated with *T. yunnanense* SB8 were examined to assess the effectiveness of *T. yunnanense* SB8 as a BCA, thus offering a sustainable solution for managing this lethal disease. Therefore, understanding the effect of *Trichoderma* treatment on SIX gene expression could improve Fusarium wilt management, paving the way for more effective and sustainable control strategies in banana cultivation.

Materials and methods

Plant materials and fungal isolates

Three-month-old rooted tissue-cultured banana (*Musa acuminata* cv. 'Berangan') plantlets were sourced from Biolife Agriculture Farm (Penang, Malaysia). For the infection studies, 40 healthy cv. 'Berangan' plantlets each with 3 to 5 leaves, a minimum height of 20 cm, and stem diameters ranging from 1.0 to 1.5 cm, were selected.

Fusarium oxysporum f.sp. *cubense* Tropical Race 4 (FocTR4) was obtained from the Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. *Trichoderma yunnanense* SB8, previously isolated from paddy soil and demonstrated to promote plant growth (Akbari et al., 2024), was utilised in this study. Both fungal cultures were stored at −80 °C as a microconidial suspension in 30% glycerol stock solution.

In vitro antagonistic assay

The antagonistic assay was performed using a dual culture technique to evaluate the biocontrol effects of *T. yunnanense* SB8 against FocTR4. Both *T. yunnanense* SB8 and FocTR4 isolates were cultured on full-strength Difco™ Potato Dextrose Agar (PDA) (BD, France). Small agar squares, each measuring 6mm^2, of strain SB8 were placed at one edge of a PDA plate, while identically sized FocTR4 agar pieces were positioned at the opposite edge. The isolates were placed 6 cm apart on the PDA plates, with each setup replicated three times.

Following a seven-day incubation at 25 °C, the antagonistic activity of *Trichoderma* isolates against FocTR4 was assessed. The percentage inhibition of radial growth (PIRG) was determined using the formula described by Chen et al. (2021):

$$PIRG(\%) = \frac{R1 - R2}{R1} \times 100$$

where R1 represents the colony radius of FocTR4 on the control plate, and R2 represents the colony radius of FocTR4 in the dual culture assay.

Fungal culture and inoculum preparation for bioassay

FocTR4 and *T. yunnanense* SB8 isolates were grown on PDA supplemented with 50 µg/mL of streptomycin sulphate (Sigma Aldrich, Germany). The plates were incubated in dark condition at 25 ± 2℃ for seven days. The 7-day-old cultures were carefully cut into several pieces (1cm^3) and prepared as two separate inoculums in 1L Difco™ Potato Dextrose Broth (PDB) (BD, France). The fungal inocula were then placed on an orbital shaker at 150 rpm for 5–7 days at room temperature (25 ± 2℃) to ensure uniform growth and proper mycelial dispersion throughout the broth. After seven days of incubation, the spores were quantified using a hemocytometer, and adjusted to a final concentration of 1×10^6 spores/mL for FocTR4 and 1×10^8 spores/mL for *T. yunnanense* SB8.

Trichoderma pre-treatment and Fusarium wilt bioassay

The tissue-cultured banana plants were transplanted into pots and acclimatized in a greenhouse under ambient temperature conditions (28 ± 2 °C) for 2 weeks. A total of 20 banana plantlets were pre-treated with *T. yunnanense* SB8 for one month, with 50 mL of SB8 inoculum (1×10^8 spores/mL) applied twice at 15-day intervals.

The Fusarium wilt bioassay was conducted according to the protocol outlined by Mohd-Yusuf et al. (2019). A total of 40 'Berangan' plantlets, including the pre-treated plantlets, were carefully uprooted for inoculation. These plantlets were evenly distributed into four distinct treatment groups, each consisting of 10 plantlets, control (T1); treated with *T. yunnanense* SB8 only (T2); inoculated with FocTR4 only (T3); treated with *T. yunnanense* SB8 and further inoculated with *Foc*TR4 (T4). Prior to inoculation, plants from treatments (T3) and (T4) were uprooted and the roots were thoroughly rinsed with tap water to remove adhering soil residues. Each plant from these treatments was then immersed in Foc TR4 spore suspension (1×10^6 spores/mL), with plants from T3 and T4 placed in separate containers corresponding to their treatments, for two hours. The inoculated plants were then replanted in the original soil and arranged on a double container tray within the greenhouse. For the control group, sterile distilled water was used instead of the inoculum. The plants were irrigated every other day with tap water.

Root samples from the treated plants were collected at three different time points: 0 day, 2 weeks and 4 weeks of post-inoculation (WPI) for molecular studies. Disease assessment was carried out at the end of 4th week post-inoculation based on the visual examination of external and internal symptoms. External symptoms, such as wilting and yellowing of the leaves, were assessed using a 5-point Leaf Symptom Index (LSI) scale. For internal symptoms, infected plants were carefully uprooted, washed to remove soil residues, and their rhizomes were vertically cut in half to inspect for brownish/dark discoloration. The Rhizome Discoloration Index (RDI) was calculated based on the intensity of rhizome infection, graded on a 8-point scale (Supplementary Table S1). Both LSI and RDI scores were used to calculate the Disease Severity Index (DSI), to assess the level of susceptibility or resistance exhibited by the tested cultivar (Mak et al., 2004). The DSI was calculated using the following formula:

Gene expression analysis by quantitative real-time PCR (qPCR)

Based on the previous study by Kaliapan et al. (2024), four *SIX* genes (*SIX1, SIX6, SIX8, SIX9*) were identified as key determinants of FocTR4 virulence and therefore selected as candidate genes in this study. Their expression patterns were examined in FocTR4 under two different conditions: (i) direct infection of cv. 'Berangan' (T3), and (ii) infection of cv. 'Berangan' plants pre-treated with *T. yunnanense* SB8 (T4), at 2 and 4 WPI. At each time point, entire root system was carefully excavated, gently washed with tap water to remove soil, and followed by thorough rinsing with sterile distilled water. Roots were surface-sterilized in 0.1% (v/v) sodium hypochlorite for 1 min, rinsed three times in sterile distilled water, blotted dry with sterile filter paper, flash-frozen in liquid nitrogen, and stored at −80 °C.

Total RNA isolation

Total RNA was extracted from fungal-infected banana roots using CTAB method based on the protocol described by Al-Obaidi et al. (2010) with slight modifications. Briefly, 1 ml of CTAB buffer and 20 µl β-mercaptoethanol were pre-heated at 65ºC for 10 min and added to 0.5 g of ground root sample, followed by an equal volume of chloroform: isoamyl alcohol, CHCl3: IAA (24:1). The mixture was vortexed and centrifuged at 9400 × *g* for 15 min. The aqueous phase was carefully transferred to a 1.5 ml tube. This step was repeated twice. In the final centrifugation step, 3 M sodium acetate (NaOAc) was added to the aqueous solution at a volume of 0.1, and the tube was filled with ice-cold absolute ethanol. The mixture in the tube was thoroughly mixed and allowed to precipitate for two days at −80ºC. After two days of precipitation, the tube was centrifuged at 9400 × *g* for 30 min. The resulting RNA pellet was washed with 1 ml of 70% ice cold ethanol and centrifuged at 4ºC, 9400 × *g* for 7 min. The pellet was then air dried and resuspended with 30 µl of DEPC-treated water. The extracted RNA was stored at −80ºC prior to quantification and qualification of RNA integrity.

cDNA synthesis and Real-time PCR (qPCR)

A total of 1 µg DNase-treated RNA was used as a template for the synthesis of first-strand cDNA using Lunascript® RT Supermix Kit (NEW ENGLAND Biolabs® Inc.) following the manufacturer's instructions. The expression profiles of *SIX* genes involved in FocTR4 infection were assessed using cDNA obtained from fungal-infected banana root samples from treatment 3 (plants inoculated in FocTR4 only) and treatment 4 (plants treated with *Trichoderma* and inoculated with FocTR4). The fungal-specific housekeeping genes, translation elongation factor 1a (*TEF1α*) and beta-tubulin (*TUB*) were used as reference genes. Real-time PCR was conducted on QuantStudio™ 12 K Flex system (Applied Biosystems, USA) using specific primers (Supplementary Table S3). The primers used for The reaction mixture included 1 µl of cDNA (1 µg/µl), 10 µl qPCRBIO SyGreen Blue Mix (PCR Biosystems, USA), 0.8µL each of 10 µM qPCR primers and 7.4µL of nuclease-free water. The geometric mean of the Ct values for the fungal-specific housekeeping genes was used for normalization of *SIX* gene expression and 0-day post inoculation root samples served as the calibrator. The thermocycling conditions for qPCR reactions were 2 min at 95ºC; 40 cycles of 5 s at 95ºC followed by 30 s at 60ºC. The relative gene expression levels of the *SIX* genes were calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Statistical analysis

The data generated from gene expression analysis were subjected to statistical analysis of one-way ANOVA and Tukey HSD post-hoc test. The significance level for all statistical tests was set at the 95% confidence interval ($p < 0.05$). The analyses were performed using IBM SPSS Statistics 26.0 software.

Results

In vitro antagonistic activity of *T. yunnanense* SB8 against *Foc*TR4

In the *in vitro* antagonistic assay, *T. yunnanense* SB8 was evaluated for its biocontrol efficacy against *Foc*TR4 using the dual culture technique in three

replicates. Notable changes in their inhibitory action towards *Foc*TR4 were observed between the seventh- and ninth-day after inoculation on PDA plates (Fig. 1). The recorded percentage inhibition of radial growth (PIRG) values for each SB8 replicate were 87.67%, 89.04% and 84.93%, with an average PIRG of 87.21%. These findings indicate that *T. yunnanense* SB8 exhibits a strong antagonistic ability against FocTR4.

Disease assessment on cv. 'Berangan' plants

Assessment of disease severity on cv. 'Berangan' plants was conducted using the Fusarium wilt bioassay method. The assessment examined the effects of *T. yunnanense* SB8 pre-treatment in reducing disease severity when plants were subsequently inoculated with FocTR4. The evaluation of disease progression was performed by visually examining the external symptoms of Fusarium wilt at the end of the 4th week post-inoculation.

Notably, the FocTR4-infected plants exhibited more yellowing symptoms compared to those that were pre-treated with *T. yunnanense* SB8 and subsequently inoculated with FocTR4. Additionally, when examining the internal symptoms, the rhizomes of the FocTR4-infected plants showed more discoloration compared to those pre-treated with *T. yunnanense* SB8. In contrast, the plants treated with sterile distilled water remained symptomless throughout the bioassay experiment. Figure 2 presents a cross-sectional view of the representative

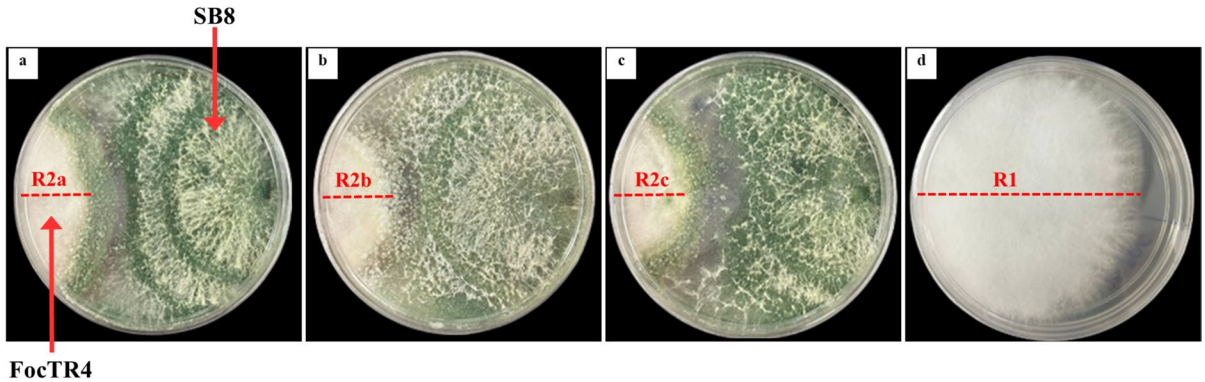

Fig. 1 *Trichoderma yunnanense* (SB8) against *Foc*TR4 in dual culture plates. (a) Replicate 1; (b) Replicate 2; (c) Replicate 3; (d) Control (*Foc*TR4)

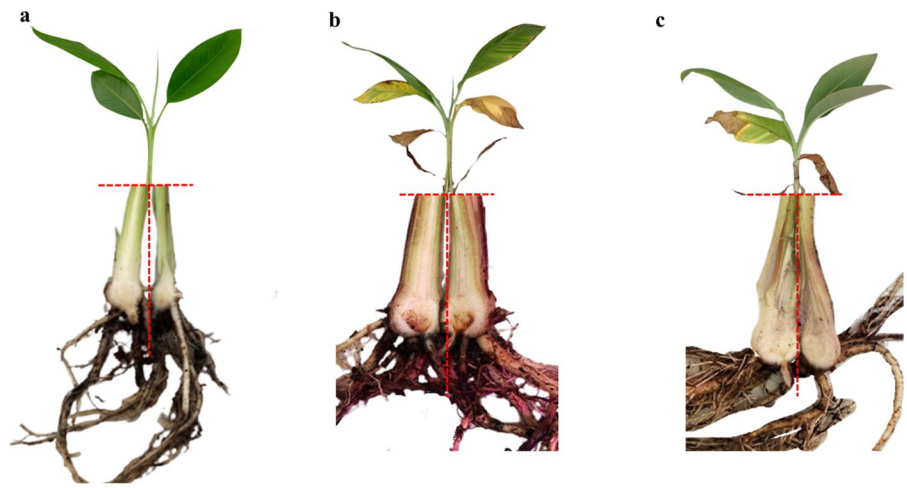

Fig. 2 Cross-sectional view of representative banana plantlets treated with different inoculums after 4 weeks post-inoculation (WPI). (a) Control plant (T1) (b) FocTR4-infected plant (T3) (c) Pre-treated with *T. yunnanense* SB8 and subsequently inoculated with FocTR4 plant (T4)

banana plantlets treated with different inoculums, illustrating the differences in disease symptoms between plants pre-treated with *T. yunnanense* SB8 and those directly inoculated with *Foc*TR4.

The DSI scores were calculated based on data obtained from the LSI and RDI (Supplementary Table S1 and S2) and summarized in Table 1. Final interpretation showed that the plants inoculated with *Foc*TR4 had a higher DSI score compared to those pre-treated with *T. yunnanense* SB8 (Table 1). This finding suggests that treatment with *T. yunnanense* SB8 suppresses the development of Fusarium wilt disease in the susceptible cv. 'Berangan' plants.

Analysis of secreted in xylem (SIX) gene expression in FocTR4 during infection in cv. 'Berangan' plants

qPCR analysis revealed a significant upregulation of *SIX* genes in plants directly inoculated with FocTR4 (T3), with high expression observed at the 2nd week post inoculation. Among these, *SIX1i* gene exhibited highest expression (4.2-fold) (Fig. 3a), followed by *SIX9a* (3.0-fold) (Fig. 3d), *SIX8a* (2.7-fold) (Fig. 3c), and *SIX6* (2.4-fold) (Fig. 3b). On the other hand, plants pre-treated with SB8 (T4) demonstrated a similar temporal expression pattern across all *SIX* genes (*SIX1i, SIX6, SIX8a* and *SIX9a*), but at a comparatively lower levels (Fig. 3a-3d), indicating that

Table 1 A summary of the Disease Severity Index (DSI) scores and the final interpretation of cv. 'Berangan' plants after 5 weeks post-inoculation (WPI)

Treatment	Index	Disease Severity Index (DSI)	Final interpretation
*Foc*TR4	Leaf Symptom Index (LSI)	4.0	Highly susceptible
	Rhizome Discoloration Index (RDI)	5.0	
T. yunnanense SB8 + *Foc*TR4	Leaf Symptom Index (LSI)	3.0	Susceptible
	Rhizome Discoloration Index (RDI)	3.0	

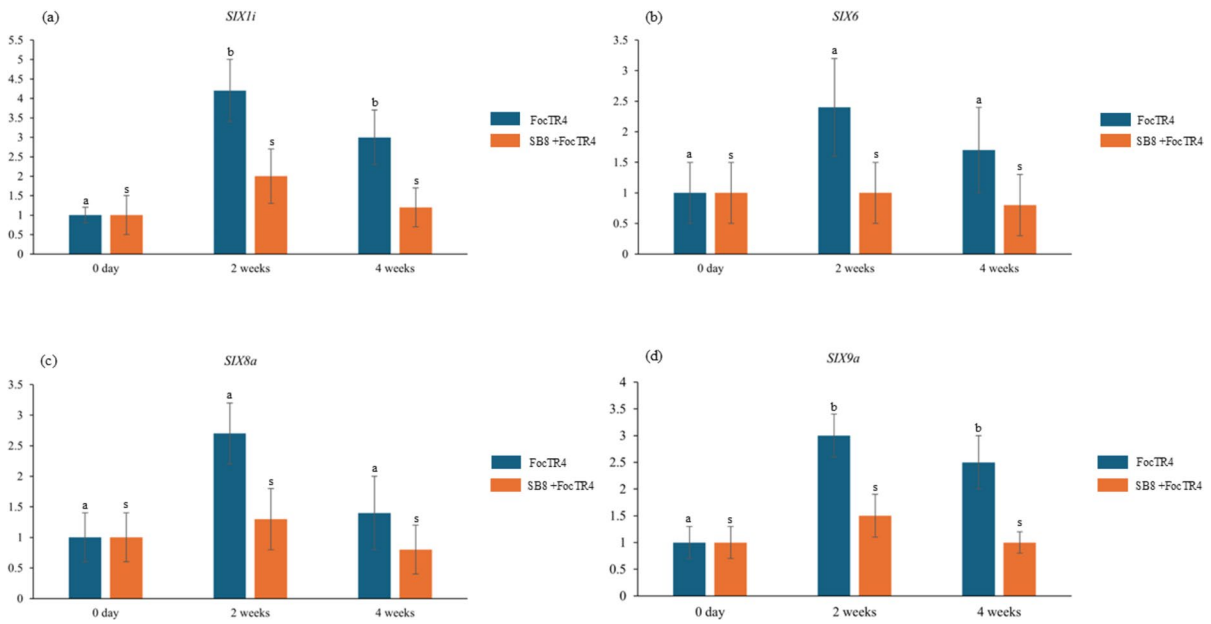

Fig. 3 The fold changes in the expression of *SIX* genes (a) *SIX1i* (b) *SIX6* (c) *SIX8a* (d) *SIX9a* in cv. 'Berangan' plants inoculated with *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 (FocTR4) and cv. 'Berangan' plants pre-treated with *T. yunnanense* SB8 at different time points (2 weeks and 4 weeks). 0-day plants served as the control for this experiment. Error bars represent the standard deviation of the results. The letters indicate significant differences (One-way ANOVA, Tukey HSD comparison test $p < 0.05$)

SB8 pre-treatment suppresses the expression of these effector genes compared to plants directly inoculated with FocTR4.

Discussion

Microbial biocontrol agents are widely used as alternative methods for controlling plant pathogens, as chemical approaches, while effective, can lead to environmental contamination (Tawfeeq Al-Ani et al., 2020). Among fungal groups, the genus *Trichoderma* has been widely studied for its role in reducing plant diseases (Adnan et al., 2019; Asad, 2022; Tyśkiewicz et al., 2022). *Trichoderma* spp. can antagonize, parasitize, or even kill other fungi, making them as a valuable agent for the biological control of phytopathogenic fungi (Harman et al., 2004). In this study, we investigated the antagonistic ability of *T. yunnanense* SB8 against FocTR4. The *in vitro* dual culture assay revealed a growth inhibition of ~90% on the pathogen, indicating its strong antagonistic potential.

Consistently, *in vivo* assessment demonstrated high virulence of *Foc*TR4, as indicated by LSI and RDI values, which is most likely associated with the presence of SIX effector genes. However, pre-treatment with *T. yunnanense* SB8 reduced the severity of infection, suggesting its potential to suppress pathogen aggressiveness *in planta*. This suppression could be attributed to the antagonistic activity of the biocontrol agent which modulates the expression of the *SIX* genes and mitigates disease progression. This is because, pre-treatment with *Trichoderma* not only facilitates early rhizosphere colonization but also primes host defence mechanisms, conferring a competitive advantage against pathogen invasion (Gupta & Bar, 2020).

To substantiate these effects, the expression of selected virulent *SIX* genes was investigated to evaluate whether *T. yunnanense* SB8 influences their regulation during host–pathogen interactions under two different time points. In plants inoculated with *Foc*TR4, *SIX1i, SIX6, SIX8a* and *SIX9a* were upregulated on the 2nd week post-inoculation. The increased expression levels benefit the fungal pathogen during the early stages of infection. Previous research by Kaliapan et al. (2024) identified the 2nd week as a critical time point for early disease detection via *SIX* gene expression analysis. This timeframe captures the hemibiotrophic transition of *Fusarium oxysporum* from biotrophy to necrotrophy (Pradhan et al., 2021), driven by escalating host defenses and the pathogen's nutrient demands for aggressive proliferation (Kabbage et al., 2015; Prigigallo et al., 2022). Therefore, during this period, the pathogen employs stealthy strategies to evade host defenses while establishing colonization (0 day and 2nd week), and subsequently causing rapid cell death in hosts while triggering significant molecular responses from the plant (4th week). Since the visible Fusarium wilt symptoms typically emerge 2–6 months after root infection (Pegg et al., 2019; Ploetz, 2015), early molecular diagnostics at 2–4 WPI provides valuable insights into pathogen detection and biocontrol efficacy.

Here, the challenge lies in determining whether BCAs can reduce the increased expression during these time points. This study suggests that pre-treatment with BCAs, specifically *T. yunnanense* SB8, may offer a solution. Plants pre-treated with *T. yunnanense* SB8 showed a reduction in the *SIX* gene expression levels at the critical time points, demonstrating the efficacy of BCAs against the fungal pathogen. In a previous study, Gomes et al. (2017) demonstrated that *Trichoderma harzianum* modulates the *in vitro* expression of virulence-associated genes in *Botrytis cinerea*, the causal agent of grey mould in tomato. Furthermore, their findings revealed that *T. harzianum* induced the upregulation of tomato defence-related genes, particularly those associated with the salicylic acid (SA)-mediated signalling pathway. Similarly, another study by Taribuka et al. (2017) demonstrated that treatment with *T. harzianum* induced lignification in banana plant tissues, where the reinforcement of cell walls restricted pathogen spread from infected to healthy tissues, thereby mitigating disease severity. Collectively, these studies highlight the multifaceted mechanisms where *T. harzianum* suppresses pathogen virulence and strengthens host defence systems.

Taken together, this study revealed that *T. yunnanense* SB8 inhibited the growth of FocTR4 *in vitro* and reduced its virulence *in planta*. This suppression is likely due to SB8's antagonistic activity, which enhances the plants resilience to FocTR4 infection. Building on the current findings, future research should investigate the expression of key

plant defense-related genes in response to *T. yunnanense* SB8 pre-treatment. Such analyses would provide more comprehensive understanding of the host–pathogen–biocontrol agent interaction and elucidate how SB8 enhances host resistance at the molecular level. Hence, this would further support the implementation of biocontrol agents, particularly *T. yunnanense* SB8-based treatments as an effective and sustainable strategy to reduce the impact of virulent pathogens such as *Foc*TR4.

Conclusion

In conclusion, this study demonstrated that *T. yunnanense* SB8 strain is a promising biocontrol agent for the effective management of Fusarium wilt disease. The strain's inhibitory properties against *Foc*TR4 highlights its potential to reduce the impact of this pathogen on banana crops. However, further research is required to optimise the application methods particularly in large scale plantations to fully harness the benefits of the *T. yunnanense* SB8 against *Foc*TR4. These findings provide a foundation for improved management strategies, thereby supporting sustainable production, promoting economic growth and enhancing food security.

Acknowledgements The authors jointly acknowledge the Institute of Biological Sciences, Faculty of Science and the Centre for Research in Biotechnology for Agriculture (CEBAR), Universiti Malaya, Malaysia.

Author contributions Conceptualization: NSMS, YMY and KK. Writing and original draft preparation: KK. Writing-Review & Editing: NSMS, YMY, FD, NBS and SNZ. Visualization: KK and SNZ. Supervision: NSMS and YMY. All authors read and approved the final manuscript.

Funding Open access funding provided by The Ministry of Higher Education Malaysia and Universiti Malaya. This work was supported by the Universiti Malaya, Malaysia through UM International Collaboration Grant (grant number ST086-2022) awarded to NSMS.

Data Availability The datasets generated during the current study are available from the corresponding author upon request.

Declarations

Conflict of interest The authors declare no conflict of interest.

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