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Fermentation of *Foc* TR4-infected bananas and *Trichoderma* spp

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ABSTRACT. Fusarium wilt (also known as Panama disease) is one of the most destructive banana diseases, and greatly hampers the global production of bananas. Consequently, it has been very detrimental to the Chinese banana industry. An infected plant is one of the major causes of the spread of Fusarium wilt to nearby regions. It is essential to develop an efficient and environmentally sustainable disease control method to restrict the spread of Fusarium wilt. We isolated *Trichoderma* spp from the rhizosphere soil, roots, and pseudostems of banana plants that showed Fusarium wilt symptoms in the infected areas. Their cellulase activities were measured by endoglucanase activity,

β-glucosidase activity, and filter paper activity assays. Safety analyses of the *Trichoderma* isolates were conducted by inoculating them into banana plantlets. The antagonistic effects of the *Trichoderma* spp on the *Fusarium* pathogen *Foc* tropical Race 4 (*Foc* TR4) were tested by the dual culture technique. Four isolates that had high cellulase activity, no observable pathogenicity to banana plants, and high antagonistic capability were identified. The isolates were used to biodegrade diseased banana plants infected with GFP-tagged *Foc* TR4, and the compost was tested for biological control of the infectious agent; the results showed that the fermentation suppressed the incidence of wilt and killed the pathogen. This study indicates that *Trichoderma* isolates have the potential to eliminate the transmission of *Foc* TR4, and may be developed into an environmentally sustainable treatment for controlling Fusarium wilt in banana plants.

Key words: Banana; *Trichoderma*; *Foc* TR4; Fusarium wilt; Biodegradation; Disease control

INTRODUCTION

Fusarium wilt is one of the most notorious diseases that affect banana plants, and it is caused by the soil-borne hyphomycete, *Fusarium oxysporum* f. sp *cubense* (*Foc*) (Ploetz, 2015). Based on the its virulence in specific banana cultivars (Ploetz, 1990,1994), the pathogen can be classified into three races: 1, 2 and 4. In the 1950s, exports of Gros Michel bananas from Central America to North America and Europe were dramatically reduced owing to *Foc* Race 1 infection (Ploetz, 1994). *Foc* subtropical Race 4 has reduced the production area of Taiwan from 50,000 to 5000 ha in the last 10 years (Hwang and Ko, 2004); *Foc* tropical Race 4 (TR4) is the most virulent strain of *Foc*, and is presently sweeping through banana plantations in Asia. It has become a major threat to the banana industry (Li et al., 2012). The disease has changed the geographical distribution of banana-growing districts in mainland China. Ten years ago, more than 50% of Chinese banana output was from Guangdong Province, but that has now been reduced to approximately 20%; the growing area has decreased from 140,000 to 70,000 ha, and now ranks third after Guangxi and Yunnan provinces. The incidence of disease in Guangdong Province now ranges between 20 and 40%, with individual plantations reaching 90% (Huang et al., 2012). Without effective inspection and quarantine measures, this disease has spread rapidly to new growing areas.

To control the spread of *Foc* TR4, the routes of transmission must be eliminated. Infected plants transmit disease through soil, air, and flowing water. Once the spore contacts the host plant it germinates and penetrates the plant tissue, producing abundant microconidia in the xylem vessels; these move to other plant organs through the vascular system, culminating in systemic colonization of that system (Beckman, 1990). Ideally, severely affected plants should be uprooted and incinerated. In a production scenario, incineration is unfeasible owing to the huge amounts of diseased biomass in the infected areas. Burning the banana plants also results in the loss of nutrients and causes air pollution.

An environmentally sound, safe, and efficient method for processing diseased plants is urgently needed. In this study, we aimed to: 1) screen *Trichoderma* species found in the

rhizosphere soil, roots, and pseudostems of banana plants for high cellulase activity, 2) use the *Trichoderma* isolates to ferment the diseased plants, and 3) determine the biocontrol capabilities of those isolates through inactivation of the pathogen in a greenhouse.

MATERIAL AND METHODS

Isolation and identification of *Trichoderma* spp

Trichoderma spp were isolated from the rhizosphere soil, roots, and pseudostems of banana plants that showed symptoms of Fusarium wilt, collected from fields in Dongguan, Guangzhou, and Zhongshan, China. The *Trichoderma* spp were isolated from soil samples by a serial dilution technique using *Trichoderma*-selective medium. The root and pseudostem samples were processed for the isolation of endophytic *Trichoderma* according to the protocol described by Mysore et al. (2005).

Testing antagonism of *Trichoderma* spp against *Foc* TR4

A dual culture technique was performed as described by Morton and Stroube (Morton and Stroube, 1955; Joshi et al., 2010). Discs (4 cm in diameter) were cut from the growth media of *Trichoderma* spp and *Foc* TR4 using a cork borer and were placed separately on potato dextrose agar (PDA) (Shenggong, Shanghai, China) medium 1 cm from the edge of the plate. Three replicate plates for each treatment were maintained. Percent inhibition over control was calculated using the following formula (Joshi et al., 2010):

$$I = (C - T) \times 100\%/C$$

where I = percent inhibition in mycelia growth, C = growth of pathogen on control plates, and T = growth of pathogen on dual culture plates.

Measurement of enzyme activity

Filter paper activity assays were performed to determine the total cellulase activity of each *Trichoderma* isolate according to the method described by Ghose (1987). *Trichoderma* isolates were cultured and filtered to prepare appropriate dilutions to provide the enzyme source. Dilute culture (0.5 mL) was mixed with 1 mL citrate buffer (0.1 M, pH 4.8), and 50 mg Whatman No. 1 filter paper (1 x 6 cm strip) and incubated at 50°C for 1 h. The amount of reducing sugar released from the filter paper was determined by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of filter paper activity was defined as the amount of enzyme releasing 1 mmol reducing sugar from filter paper per mL per min (Gautam et al., 2012).

Endoglucanase activity was measured using a reaction mixture containing 1% carboxymethyl cellulose (1 mL) in 0.05 M sodium citrate buffer, pH 4.8, and aliquots of the diluted culture filtrate; the quantity of reducing sugar produced was determined by the DNS method (Miller, 1959). β-glucosidase activity was assayed using the protocol described by Pointing et al. (1999). One unit of enzyme activity was defined as the amount of enzyme releasing 1 mmol reducing sugar per min.

Safety analysis of the *Trichoderma* isolates on banana plantlets

PDA Petri plates containing the *Trichoderma* isolates and *Foc* TR4 were incubated at 28°C until they were well sporulated, and were then stored at 4°C. The spores were harvested by washing the Petri plates with 50 mL double-distilled water (ddH_2O) containing 0.1% Tween-80 (Sigma-Aldrich). The spore concentration was determined in a counting chamber and the inoculum concentration was adjusted to 1.0 x 10^6 CFU/mL. We applied a modified version of the stab-inoculation method described by Haygood and Strider (1982). Brazilian banana plantlets (Musa AAA Cavendish) were produced by tissue culture in our group facility, and plantlets with four or five leaves were selected for the inoculation studies. The spores (5.0 μL) were stab-inoculated into the wounded pseudostems; water and *Foc* TR4 spores served as the negative and positive controls, respectively.

Fungal identification

Trichoderma isolates with high cellulase activity, no observable pathogenicity to banana plants, and high antagonistic capacity were identified according to the protocol developed by Castle et al. (1998). Colony appearance and sporulation patterns were recorded from cultures grown on PDA or cornmeal dextrose agar at 25°C in darkness. Morphological characteristics were recorded under a Zeiss Axiophot 2 microscope with an Axiocam CCD camera and Axiovision digital imaging software (Axiovision Software Release 3.1., v.3-2002; Carl Zeiss Vision Imaging System).

The DNA of four *Trichoderma* isolates was extracted using an E5038 plant/fungus DNA isolation kit (Sigma). Genomic regions of the three genes, i.e., translation-elongation factor 1 alpha (*tef1*), RNA polymerase II subunit B (*rpb2*), and ATP citrate lyase (*acl1*) were amplified following the procedure described by Jaklitsch et al. (2013), and the polymerase chain reaction products were extracted from 1.5% agarose gel and sequenced by the Beijing Genomics Institute. Subsequently, sequences (*tef1*, *rpb2*, and *acl1*) were BLASTed against the National Center for Biotechnology Information (NCBI) database, using the default parameter. Thirty-five homologous sequences were downloaded from the different *Trichoderma* spp based on high sequence similarity. All 35 homologs and the four reference sequences from each gene were aligned separately using the Molecular Evolutionary Genetics Analysis Version 6.0 (MEGA6) software and manually trimmed. The trimmed sequences of each gene were then joined together using an ad hoc perl script written in-house. The homologs and reference sequences were then aligned again with MEGA6 using the default parameter (Tamura et al., 2013). A neighbor-joining bootstrap phylogeny tree was constructed using the default parameter, and the bootstrap replication number was 1000.

Single-spore cultures of the identified *Trichoderma* isolates were frozen at -80°C in 50% glycerol, and are archived in the Agricultural Culture Collection of China in the Chinese Academy of Agricultural Sciences, Beijing.

Biodegradation of the diseased banana plants and bioassays

Initial cultivation of Trichoderma isolates

The defined initial cultivation medium contained 70% dried and milled banana

stalks, 30% wheat bran, 2% ammonium sulfate solution (100 mL with 0.1 g KH_2PO_4 and 0.05 g $MgSO_4 \cdot 7H_2O$); the media was autoclaved at 121°C for 20 min and cooled. The spore suspensions of the *Trichoderma* isolates with high cellulolytic activity were inoculated at a rate of 10^6 spores/g medium, and incubated for 10 days at 28°C.

Small-scale fermentation and greenhouse test

A green fluorescent protein (GFP)-tagged *Foc* TR4 spore suspension was prepared as described previously. The artificial inoculation of banana plants for small-scale fermentation and biocontrol testing in a greenhouse has been described previously (Li et al., 2011), except that the spore concentration in the soil was increased to 10,000 conidia/g soil and 400 Brazilian banana plants (*Musa* spp, AAA-group) with 10-12 leaves were inoculated. After 1 month, the diseased banana plants were harvested and cut into pieces; they were then mixed with equal amounts of rice straw and 1% *Trichoderma* complex for fermentation. The composting materials were covered with plastic films and fermented for 30 days. After fermentation, the samples were examined by fluorescence microscopy (Li et al., 2011) and incubated in PDA medium to assay for pathogen survival.

The fermented composts were tested for biocontrol of the pathogenic agent. The soil (Li et al., 2011) was autoclaved and mixed with 20% of the fermented compost; the GFP-tagged *Foc* TR4 spores were then added to the soil to a final concentration of 2000 spores/g soil. Two controls were used: the positive control contained soil and fermentation product without the inoculated pathogen, whereas the negative control contained the pathogen in the soil and no fermentation product. After plantlets with 5-6 leaves had been transplanted, the pots were maintained in the greenhouse at ~30°C for 30 days. The plants were then removed from the soil and the roots were washed with sterile distilled water. The roots were excised from the plants and the data were collected for analysis.

RESULTS

Screening strains of *Trichoderma* spp

The potential use of *Trichoderma* species as biocontrol agents against crop diseases caused by *Fusarium* has been reported (Sivan et al., 1987; Sundaramoorthy and Balabaskar, 2013). In the present investigation, 221, 45, and 9 strains of *Trichoderma* spp were isolated from the rhizosphere soil, roots, and pseudostems of banana plants, respectively. Of the isolates tested, four strains (TM1, 2, 3, and 4) were selected for further research including biofermentation and biocontrol tests in the greenhouse, owing to their strong antagonistic effect on *Foc* TR4 (Table 1 and Figure 1), high cellulolytic activity, and absence of disease symptoms on the stab-inoculated pseudostems of banana plantlets (Table 1 and Figure 2). The four isolates were characterized by their conidiophores, phialide phenotypes (among others), and the results of phylogenetic analysis, and they were determined to be *Trichoderma polysporum*, *T. longipile*, *T. viride*, and *T. harzianum* (Table 1 and Figure 3).

Table 1. Characteristics of the four *Trichoderma* isolates.

Items		TM1	TM2	TM3	TM4
Source		Root	Soil	Soil	Soil
Conidiophores	Length (μm)	2.7-3.8	3.9-6.9	3.3-4.2	2.2-3.8
	Width (μm)	1.4-2.3	2.8-4.5	3.1-3.8	2.4-3.3
	R1	1.2-2.0	1.0-1.6	1.0-1.3	1.0-1.5
Phialides	Length (μm)	3.9-6.2	8.4-18.1	5.9-11.4	5.6-9.4
	Maximum width (μm)	3.1-3.8	3.0-3.5	2.1-3.4	2.5-3.4
	Base width	2.0-2.9	1.7-2.8	1.0-2.8	1.6-2.8
	R2	1.2-1.9	2.3-5.1	1.9-3.9	1.5-2.2
Colony color		White	Green-yellow	Green-yellow	Green
Rate of inhibition		64.56%	58.54%	60.52%	70.68%
Enzyme activity (IU/mL)	Exoglucanase	2.16 ± 0.05	1.76 ± 0.04	2.05 ± 0.05	1.66 ± 0.04
	Endoglucanase	2.01 ± 0.04	1.78 ± 0.08	1.79 ± 0.06	1.46 ± 0.04
	β-glucosidase	1.93 ± 0.03	1.69 ± 0.03	1.96 ± 0.09	1.42 ± 0.06

R1: length/width. R2: length/maximum width. TM: *Trichoderma.*

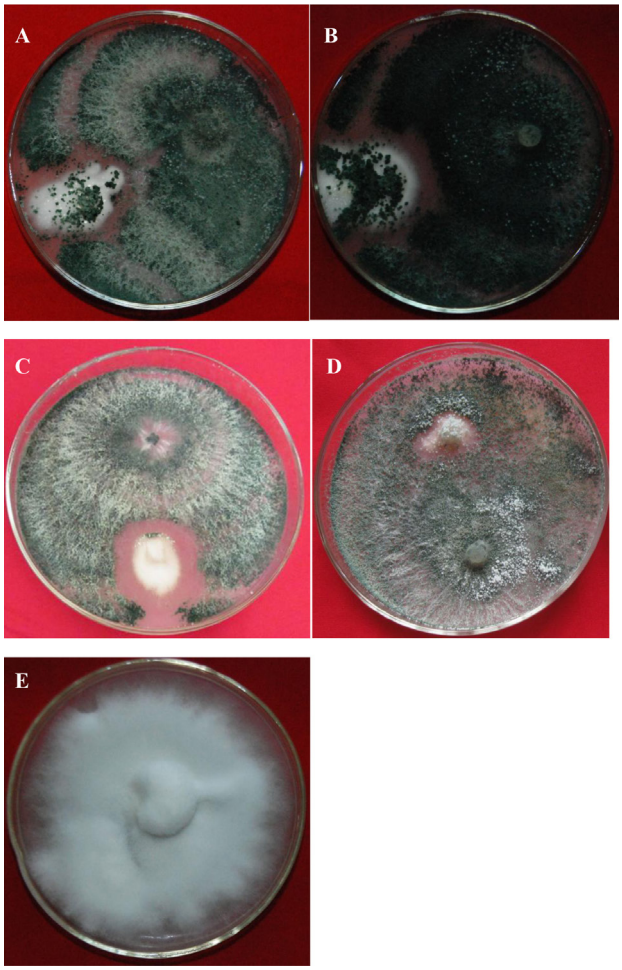

Figure 1. Antagonistic efficacy of *Trichoderma* spp against *Foc* TR4 under *in vitro* conditions. **A.** TM1; **B.** TM2; **C.** TM3; **D.** TM4; **E.** *Foc* TR4.

Figure 2. Safety analysis of the *Trichoderma* isolates on banana plantlets. **A.** Pathogenic *Trichoderma* isolates showed spreading disease symptoms; **B.** biocontrol *Trichoderma* isolates and water showed the symptoms of programmed cell death.

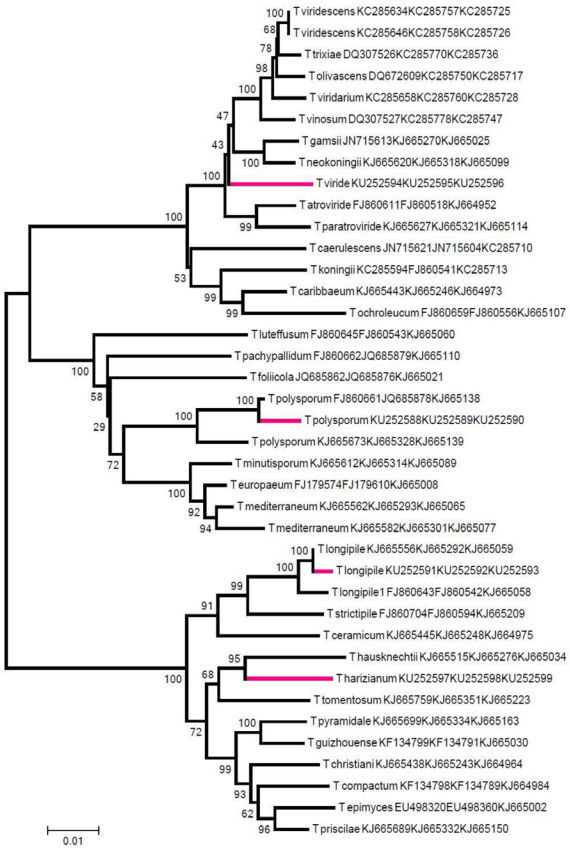

Figure 3. A phylogenetic tree based on combined *tef1*, *rpb2*, and *acl1* sequences. Bootstrap values ≥ 50% are shown above or below branches. Each taxon contains the isolate name and the GenBank accession No. (*tef1*, *rpb2*, and *acl1*); the four identified *Trichoderma* isolates are marked in red.

Small-scale fermentation

The GFP-tagged *Foc* TR4 isolate invaded the susceptible Cavendish banana cultivar and after 1 month the plants showed the characteristics of Fusarium wilt disease, including root rot, yellow leaves, and vascular discoloration. Confocal laser scanning microscopy of banana pseudostems on day 30 showed considerable colonization by the GFP-tagged *Foc* TR4 isolate, with fungal hyphae and spores covering large sections of the vascular tissue (Figure 4).

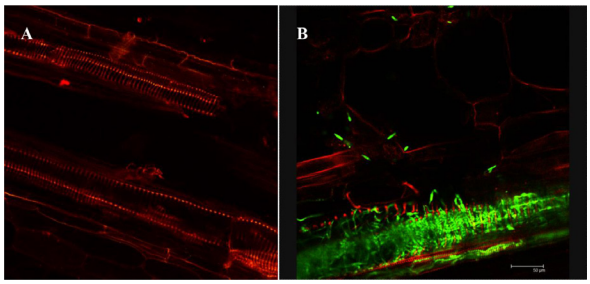

Figure 4. Invasion of green fluorescent protein (GFP)-tagged *Foc* TR4 isolate in susceptible banana vascular tissues. **A.** Healthy vascular tissues without *Foc* TR4; **B.** fungal hyphae and spores inside banana vascular tissues.

All four *Trichoderma* isolates grew very well in the initial cultivation medium. After 10 days, we tested the concentration of spores in the initial fermentation, which was at least 10^9 CFU in all cases. These initial fermentation cultures were mixed in equal amounts. The chopped banana pseudostems and leaves (without any dry processing) were mixed with the *Trichoderma* complex described above and rice straw, and were fermented for 30 days. After 3 days of fermentation, a large amount of stem sap flowed out; the control, which consisted of chopped banana pseudostems and rice straw without the *Trichoderma* complex, did not exhibit this phenomenon (Figure 5). The temperature of the *Trichoderma* treatment gradually increased throughout the process of fermentation, and the highest temperature inside the compost (50°C) was reached after 20 days. After 30 days, the compost cooled to ambient temperature. The control reached a maximum temperature of only 35°C after 25 days.

Figure 5. Compost of banana pseudostems and leaves with *Trichoderma* isolates and the control. **A.** Control; **B.** compost of banana pseudostems and leaves fermented with the *Trichoderma* isolates.

After 30 days of fermentation, we could not discern the clear cell structure of the composted materials and the GFP-tagged *Foc* TR4 spores in the *Trichoderma* treatment. The GFP-tagged spores were found in the solution of ddH_2O soaking the compost materials of the control, and many GFP-tagged *Foc* TR4 clones were obtained when inoculating back to the PDA medium; we were unable to isolate clones by inoculating the fermented *Trichoderma*

treatment, which indicates that the *Trichoderma* with high cellulolytic activity destroyed the cell structures and killed the pathogen.

We altered the fermentation conditions and investigated their efficiency by adding the four *Trichoderma* isolates to the compost individually; when compared with the complex mixture, their fermentation rates decreased, suggesting that different mechanisms were taking place and that a synergistic effect was involved in the process. Removal of the rice straw from the compost not only slowed the fermentation rates, but also resulted in a marked decrease in the number of *Trichoderma* spores; this indicates that rice straw could improve *Trichoderma* propagation and accelerate its colonization.

Bioassay

Application of the *Trichoderma* compost to the soil was effective in reducing the occurrence of Fusarium wilt in banana plants by 30.08-63.91% (Table 2). All the single *Trichoderma* composts TM1, 2, 3, and 4 decreased the incidence of wilt by 51.18, 37.66, 30.08, and 45.51%, respectively; notably, adding the complex *Trichoderma* compost to the soil decreased the incidence of wilt by 63.91%, which suggests that it had a synergistic effect in suppressing the invasion of *Foc* TR4, and a combination of several strains with different biocontrol mechanisms might be exploited in sustainable disease management programs.

Table 2. Efficacy of *Trichoderma* bioformulation in the management of Fusarium wilt in banana plants under greenhouse conditions.

Trt. No.	Fungal antagonists	Plant height (cm)*	Percent disease incidence*
1	TM1 compost	14.18^{a}	25.27^{d}
2	TM2 compost	11.59^{b}	38.79^{e}
3	TM3 compost	11.06^{c}	46.37^{f}
4	TM4 compost	11.47^{b}	30.94^{d}
5	Complex compost	13.94^{a}	12.54^{c}
6	Negative control1	10.35^{d}	76.45^{h}
7	Negative control2	10.22^{d}	64.38^{g}
8	Positive control	11.36^{b}	0^{a}
9	Complex control	14.02^{a}	0^{a}
10	TM1 control	14.39^{a}	0^{a}
11	TM2 control	11.43^{b}	5.12^{b}
12	TM3 control	11.21^{b}	10.16^{c}
13	TM4 control	11.33^{b}	5.62^{b}

*Mean of three replications. The same letters in the same column were not significantly different at $P < 0.01$ by the Duncan multiple range test. TM1, 2, 3, 4, and complex control: only compost and without pathogen inoculated into the soil.

Positive control: the natural compost of pieced banana pesodostems and rice straw, without the *Trichoderma* isolates and *Foc* TR4. Negative control 1and 2: the natural compost of pieced banana pesodostems and rice straw, without the *Trichoderma* isolates, inoculated with *Foc* TR4 in the soil.

The results of this experiment also revealed that the application of fermentation products from TM1 and the complex antagonistic fungal formulations significantly increased banana plant height when compared with those from other isolates of *Trichoderma* and both controls (Table 2). Because TM1 was isolated from banana plant roots, we can deduce that endophytic fungi promoted plant growth. Although we have not yet investigated the mechanism

of plant growth promotion, it may be associated with the secretion of auxins, gibberellins, and cytokinins (Waqas et al., 2012).

The pot experiments showed that upon treatment with TM1 and complex *Trichoderma* composts, the *Foc* incidence rate was 0, which suggests that both treatments completely killed the pathogen within the fermentation materials; this was consistent with our confocal microscopy observations. A few diseased plants with wilt symptoms were found in the three other *Trichoderma* compost treatments; however, the incidence was very low compared with the negative control (Table 2). The only difference between the treatment and control was the presence of *Trichoderma* isolates in the fermentation compost, which suggests that most of the pathogen spores were killed by fermentation with the other *Trichoderma* isolates; the GFP-tagged *Foc* TR4 was undetectable by confocal microscopy.

DISCUSSION

In this study, the four fungal antagonist isolates, particularly the complex, had high cellulolytic activity and biomass conversion rates. All the *Trichoderma* isolates (particularly the complex formulation) had excellent biocontrol capabilities, and could be used to safely process the great mass of diseased plants in a field setting. However, fermentation of banana pseudostems in the field using the *Trichoderma* isolates described above is time-consuming, which is a disadvantage for farmers. Our next research objective will be to speed up this fermentation process (Zhang and Yang, 2015), improve the inoculum application method, conduct co-inoculation with different microbial strains, and determine how those modifications affect the biological control of Fusarium wilt (Raza et al., 2016).

Conflicts of interest

The authors declare no conflict of interest.

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