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Full Length Research Paper

Trichoderma spp. from rhizosphere soil and their antagonism against *Fusarium sambucinum*

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One hundred and forty six (146) isolates of *Trichoderma* spp. were obtained from rhizosphere soils of potato plants in the middle areas of Gansu Province, China. By means of dual culture method, they were examined for antagonism against *Fusarium sambucinum*, which causes potato dry rot. Ten of the isolates were found to be evidently antagonistic to the pathogen. Based on morphological characteristics and molecular analyses, the antagonistic isolates were identified as *Trichoderma harzianum* Rifai, *Trichoderma longibrachiatum* Rifai, *Trichoderma atroviride* Karsten and *Trichoderma virens*. Among these isolates, D-3-1 (*T. longibrachiatum*) showed the strongest inhibition of the growth of *Fusarium sambucinum*.

Key words: *Trichoderma*, potato, dry rot, biological control, *Fusarium sambucinum*.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is the fourth important crop in the world, apart from wheat, rice and maize (Li et al., Fa). China is the largest country in the world that produces potato, and this plant plays an important role in the national economy. Diseases constitute the main limiting factors in potato production.

Pathogenic viruses, bacteria and fungi can decrease potato quality and yield.

Potato dry rot caused by *Fusarium sambucinum* is a fungal disease that strongly affects potato yield and quality in Gansu Province, China (Li et al., 2007b; Sun et al., 2008). It is essential for scientists to develop an effective, safe and healthy way to control the dry rot disease. *Trichoderma* spp. have been repeatedly recorded as effective biological control agents in inhibiting plant pathogens (Ze et al., 2007).

These fungi behave as parasites and antagonists of many phytopathogenic fungi, thus protecting plants from diseases. So far, *Trichoderma* spp. are one of the most studied fungal biological control agents and commercially marketed as biological pesticides, biological fertilizers and soil amendments. Depending on the strain, the use of *Trichoderma* spp. in agriculture provide numerous advantages (Vinale et al., 2008): Rapidly establishing stable microbial communities in rhizosphere colonization; controlling pathogenic and competitive microflora by using a variety of mechanisms; improving root growth and plant health.

Trichoderma spp. have been used as antagonists in the biological control of many fungal plant diseases for many years (Elad et al., 1998; Elad and Kapat 1999; Xu et al., 1999; Abdel-Fattah et al., 2007). According to the researches on *Trichoderma* spp. and their mechanisms, there are five species with biological control effects. These species are *Trichoderma harzianum*, *Trichoderma viride*, *Trichoderma koningii*, *Trichoderma hamatum* and *Trichoderma longibrachiatum.*

The genus *Trichoderma* lives in many habitats, especially in the soil. The activity of *Trichoderma* spp. as antagonists of plant pathogens are influenced by temperature, humidity and interactions with other micro-organism communities. In Gansu Province, the resources of *Trichoderma* spp. are very abundant, and these fungi could be used to control the plant diseases.

The objectives of the present research were to obtain

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isolates of *Trichoderma* spp. from rhizosphere soil of potato plants and screen them in relation with the control of *F. sambucinum*, which can cause potato dry rot disease.

MATERIALS AND METHODS

Collection of soil samples and isolation of *Trichoderma* spp.

Forty six samples from potato cultivated soil were collected from April to October, 2007 in different areas of Gansu Province, China. Three centimeter of the top soil was removed and 5 subsamples were then taken at random at a depth of 20 cm for each site. The soil samples were then transferred into sterile polyethylene bags and transported to the laboratory. All subsamples from one site were combined to yield one composite sample representing the location, exposed to room temperature with a humidity degree of 50% and sieved through a mesh of 2 mm.

The soil samples were diluted into different concentration solution and vortexed well.

The supernatants were then poured into plates of PDA (potato dextrose agar, with chloramphenicol and sodium propionate) medium and incubated at 28°C. Colonies appearing on the plates were isolated and re-inoculated into a new plate. After 7 days, single spore colonies were obtained by subculturing at 28°C.

The *F. sambucinum* strain used in this study was isolated from dry rotted potatoes, and was provided by the Laboratory of Plant Pathology, Gansu Agricultural University.

Screening test for antagonism

Trichoderma spp. isolates and *F. sambucinum* were cultured in order to observe the interaction between them. They were cultivated separately for 5 days on PDA culture medium, then x-cm plugs from the colonies of *Trichoderma* spp. and *F. sambucinum* were placed in pairs 4 cm apart from each other on PDA plates. The controls consisted of pure *F. sambucinum* cultures. The experiment was performed four times; the plates were placed in an incubator at 28°C, and the development of the colonies was observed until the controls covered the whole potato dextrose agar (PDA) surface.

Statistics

The coefficient of antagonism was calculated using the following formula:

$$N = (LC-LP)/LC$$

Where, N is the coefficient of antagonism; LC is the radius of the control colony and LP is the radius of *F. sambucinum* in dual culture colony

Morphological identification of *Trichoderma* spp. isolates

The *Trichoderma* spp. isolates were cultured on PDA and WA (water agar) for 7 days at 28°C. The colonies morphology and feature were observed, which include spore morphology, length of conidiophores and conidiation. Specimens were coated with gold using an IB-3 ion coater (EIKO Engineering, Ibaraki, Japan) and examined with a scanning electron microscope (S-3000; Hitachi).

DNA extraction

Trichoderma spp. strains were grown in 100 ml potato/dextrose broth for 24 to 48 h at 28°C and shaken in a rotary shaker at 180 rpm. The mycelium was harvested by filtration through a piece of filter paper and washed with distilled water. A 100 mg fresh mycelium was homogenized with a micro-pestle in a 1.5 ml microfuge tube containing 400 µl LETS buffer (20 mM Tris/HCl, 100 mM LiCl, 10 mM EDTA and 0.5% sodium dodecyl sulphate, pH 7.8). A 500 µl aliquot of a 25 : 24 : 1 (v/v/v) mixture of pheol/ chloroform/ pentanol was added, and the tube was vortexed for 30 s and centrifuged at 12,000 rpm for 10 min. The upper portion of the aqueous phase (approximately 250 µl) was recovered, and DNA was precipitated by adding 0.5 ml cold absolute ethanol and incubated at -20°C for 10 min, dried *in vacuo*, and resuspended in 20 µl water (Raeder and Broda, 1985). DNA preparations were diluted 1/500 with water and used as templates for PCR amplification.

PCR amplification and sequencing of amplificons

Phylogenetic analysis of the 5.8S rDNA sequence with ITS-1 and ITS-2 regions was done by using the following primers (Irina et al., 2005): EF1-728F (5′-CATCGAGAAGTTCGAGAAGG-3′); EF1-986R (5′-TACTTGAAGGAACCCTTACC-3′).

Polymerase chain reactions (PCRs) were performed in 25 ml with 1 unit Takara Ex Taq DNA polymerase (Takara), 1× Ex Taq buffer (Mg^{2+} plus), 0.2 mM of dNTPs, 0.02 mM of each primer and 0.2 mg of the genomic DNA. Reactions were performed by denaturation for 5 min at 94°C followed by 30 cycles of 30 s at 94°C for 1 min at 55°C and 100 s at 72°C, with a final extension of 5 min at 72°C. Aliquots (4 ml) were analyzed by electrophoresis with 1.5% (w/v) agarose gel in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). The resulting PCR product was purified with a PCR and Gel Purification kit.

DNA fragments from *Trichoderma* spp. were sequenced by Shanghai Sangon. Sequence alignment and phylogenetic analysis were performed with DNAMAN software using the neighbor-joining method. The statistical significance of the branching order was estimated by 1000 replications of bootstrap re-sampling of the original nucleotide sequence alignments.

Nucleotide sequence accession numbers

The 5.8S rDNA sequence with the ITS-1 and ITS-2 regions determined in this study were submitted to the GenBank.

RESULTS

Isolation of *Trichoderma* spp. from the soil samples

One hundred and forty six (146) isolates of *Trichoderma* spp. were collected from rhizosphere soils around potato plants in the middle areas of Gansu Province, China. Among these, 52, 54, 23 and 17 isolates were from Dingxi, Lintao, Huichuan and Yuzhong, respectively.

After incubation for 2 days, colonies of *Trichoderma* spp. on PDA at 28°C were developed and became dark green at the center.

Screening test of the antagonism isolates

Via the screening test, 10 isolates showed the best

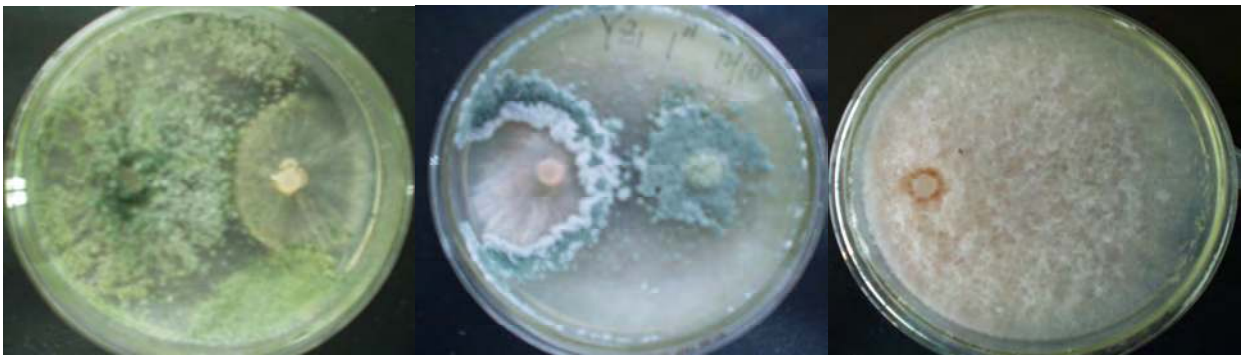

Figure 1. Inhibition of the growth of *F. sambucinum* by isolates of *Trchoderma* spp. (left: *T. longibrachitam* vs. *F .sambucinum*, center: *T. harzianum* vs. *F. sambucinum,* right: Control).

Table 1. Antagonistic indexes of *Trichoderma* isolates obtained from different potato plant soil in different sampling sites.

Number	Species	Origin	Antagonism coefficient	Genbank accession number
D-3-1	*T. longibrachiatum*	Dingxi	0.834	EU280033.1
D-4-12	*T. longibrachiatum*	Dingxi	0.829	EU401627.1
L-Z-5	*T. virens*	Lintao	0.828	EU280063.1
L-H-13	*T. harzianum*	Lintao	0.829	AF469194.1
L-D-20	*T. harzianum*	Lintao	0.828	AF348101.1
H-C-20	*T. atroviride*	Huichuan	0.828	AY376051.1
Y-Z -9	*T. harzianum*	Yuzhong	0.830	AF443947.1
Y-Z-10	*T. harzianum*	Yuzhong	0.830	AF348091.1
Y-Z-12	*T. longibrachiatum*	Yuzhong	0.828	EU401627.1
Y-Z-17	*T. longibrachiatum*	Yuzhong	0.828	EU401627.1

antagonism against *F. sambucinum* (Figure 1), the antagonism coefficients were all higher than 0.828. The results are shown in Table 1.

Identification of isolates

The 10 antagonistic isolates were identified via morphology and the genomic sequences. The 10 isolates were identified in 4 species: 4 isolates belonged to *Trichoderma hazianum,* 2 isolates belonged to *Trichoderma longibrachiatum*, 2 isolates belonged to *Trichoderma atroviride*, and 2 isolates belonged to *Trichoderma virens* (Figure 2 and Figure 4).

The colonies of *Trichoderma* isolates on PDA are shown in Figure 3. *T. hazianum* became dark green from the center, the branches system showed pyramid shape, conidia and were spherical or near spherical. *T. longibrachiatum* was light green or deep green, the conidiophores were longer than *T. hazianum*, and conidia were oval. *T. atroviride* pigmentation was dark green and the colonies smelled aromatic, conidia were spherical or near spherical, and conidiophores were more abundant and irregular. *T. virens* became dark yellow from the center, conidia were oval and the conidia were many.

PCR amplification sequencing: Phylogenetic analysis of rDNA with ITS-1 and ITS-2

Approximately, 300 bp single band was detected in polymerase chain reaction (PCR) amplifications from *Trichoderma* isolates. The large intron was contained in the 0.3 kb fragment of *tef1*. The length and sequence of the region from *Trichoderma* spp. isolates were identical to corresponding species. The phylogenetic tree based on the sequences is shown in Figure 5.

DISCUSSION

Many *Trichoderma* spp. isolates were obtained from the potato soils in the middle areas of Gansu Province, China.

According to the phylogeny system and the sequence analysis of rDNA, the 10 isolates with the best behaviour against *F. sambucinum* were identified in 4 species: *T. atrovirde*, *T. longibrachiaum*, *T. virens* and *T. hazianum*. According to their abundance in the sampled soils, *T. hazianum* and *T. longibrachiaum* were dominant among them. *Trichoderma* spp., well-known biological control agents of plant pathogens (Govindasamy and

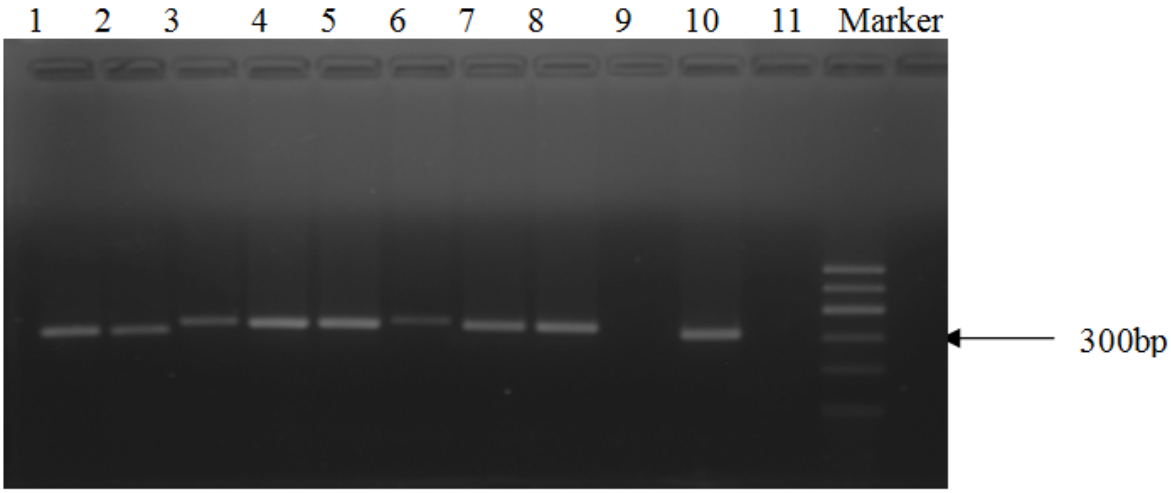

Figure 2. The bands of PCR products of 10 isolates of *Trichoderma* spp. at primer EF1. lanes 1 to 11: T-B-2, T-B-4, Y-B-10, Y-C-6, S-E-1, L-H-13, H-A-20, Y-Z-10, Y-Z-9, D-C-10, negative control respectively; M: Marker I.

Figure 3. Colony of *Trichoderma* spp. isolates on PDA (7 days), up left: *T. atrovirde*; up right: *T. longibrachiaum*; down left: *T. virens*; down right: *T. hazianum*.

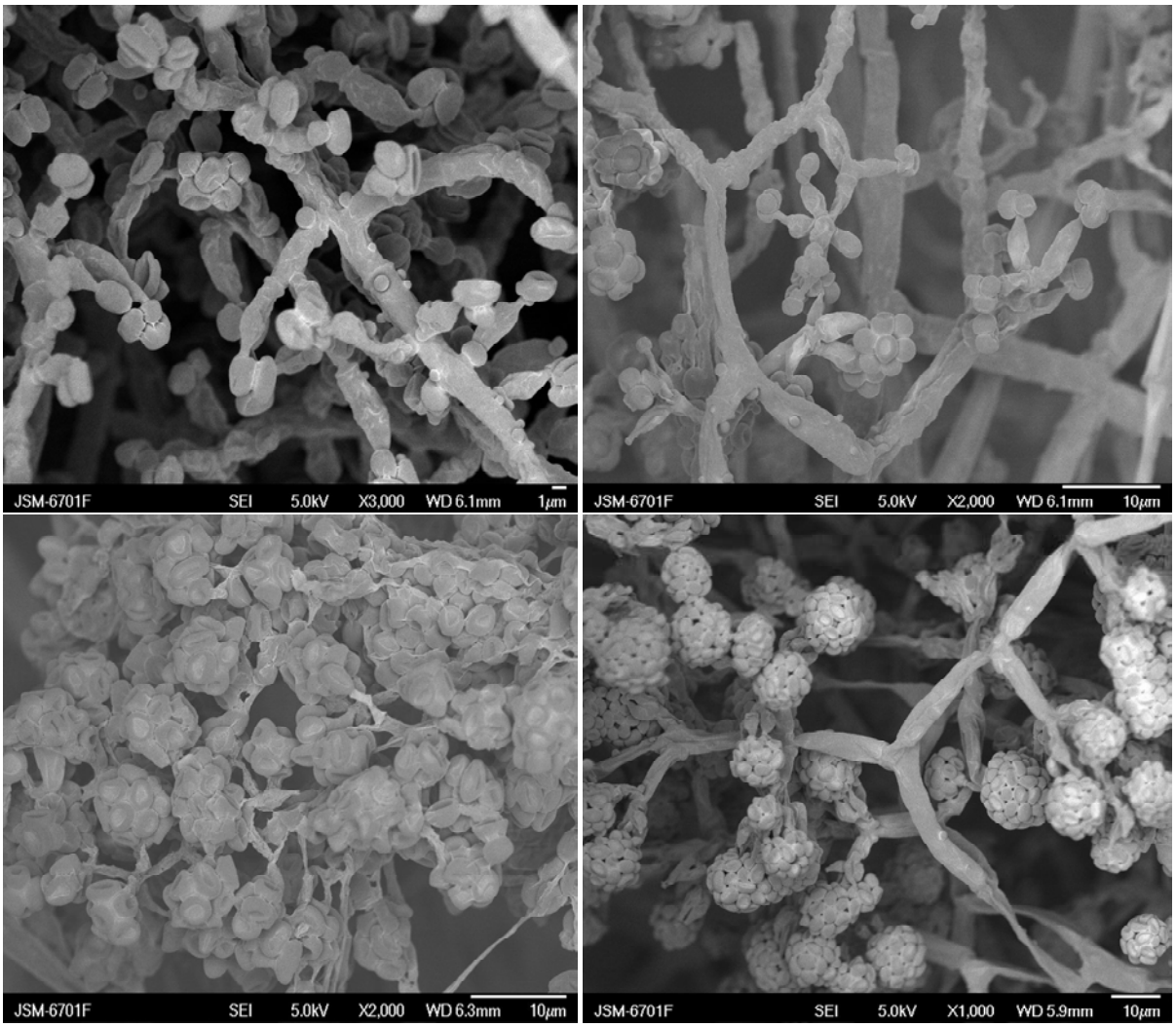

Figure 4. Conidiophores and conidia of *Trichoderma* spp. up left: *T. hazianum*; up right: *T. longibrachiaum*; bottom left: *T. atrovirde*; bottom right: *T. virens*.

Balasubramanian, 1989; Ghisalberti et al., 1990), can inhibit severaldiseases of plants. So, it seems possible that potato dry rot disease could be controlled by *Trichoderma* spp., thus leading to development in the potato industry in China (Kovach et al., 2000; Watanabe et al., 2005).

Chemical pesticides cause human health and environmental problems, which is of great concern. The application of biological control agents to inhibit the pathogens has been given great attention. As a result of this study, the antagonism of *T. longibrachiatum* against *F. sambucinum* is shown for the first time in China.

Many difficulties have arisen for the morphological identification of some *Trichoderma* spp. Other major problems have been the incorrect application of species names to isolates in industry, biological control of plant pathogens and ecological surveys, which caused questionable results comparison. In view of these reasons, DNA sequence analysis is an effective and available method to identify *Trichoderma* species. For the genus *Trichoderma*, there is a diagnostic combination of several oligonucleotides specifically allocated within the internal transcribed spacer 1 and 2 (ITS1 and ITS2) sequences of the rDNA repeat. Oligonucleotide sequences which were constant in all known ITS1 and ITS2 of *Trichoderma*, but different in closely related fungal genera were used to define genus-specific hallmark (Kullnig-Gradinger et al., 2002). The *T. atroviride* species was similar with *T. longibrachiatum* species, maybe the two species shared high homology. The result shows that *T. atroviride* maybe gotten from *T. longibrachiatum* which was firstly proposed in phylogeny and evolution of the genus *Trichoderma*.

According to the phylogenetic tree, although the isolate of D-3-1 was *T. longibrachiatum*, it had best effect on antagonism to *F. sambucinum* among the 10 isolates, which showed that this isolate was different from others.

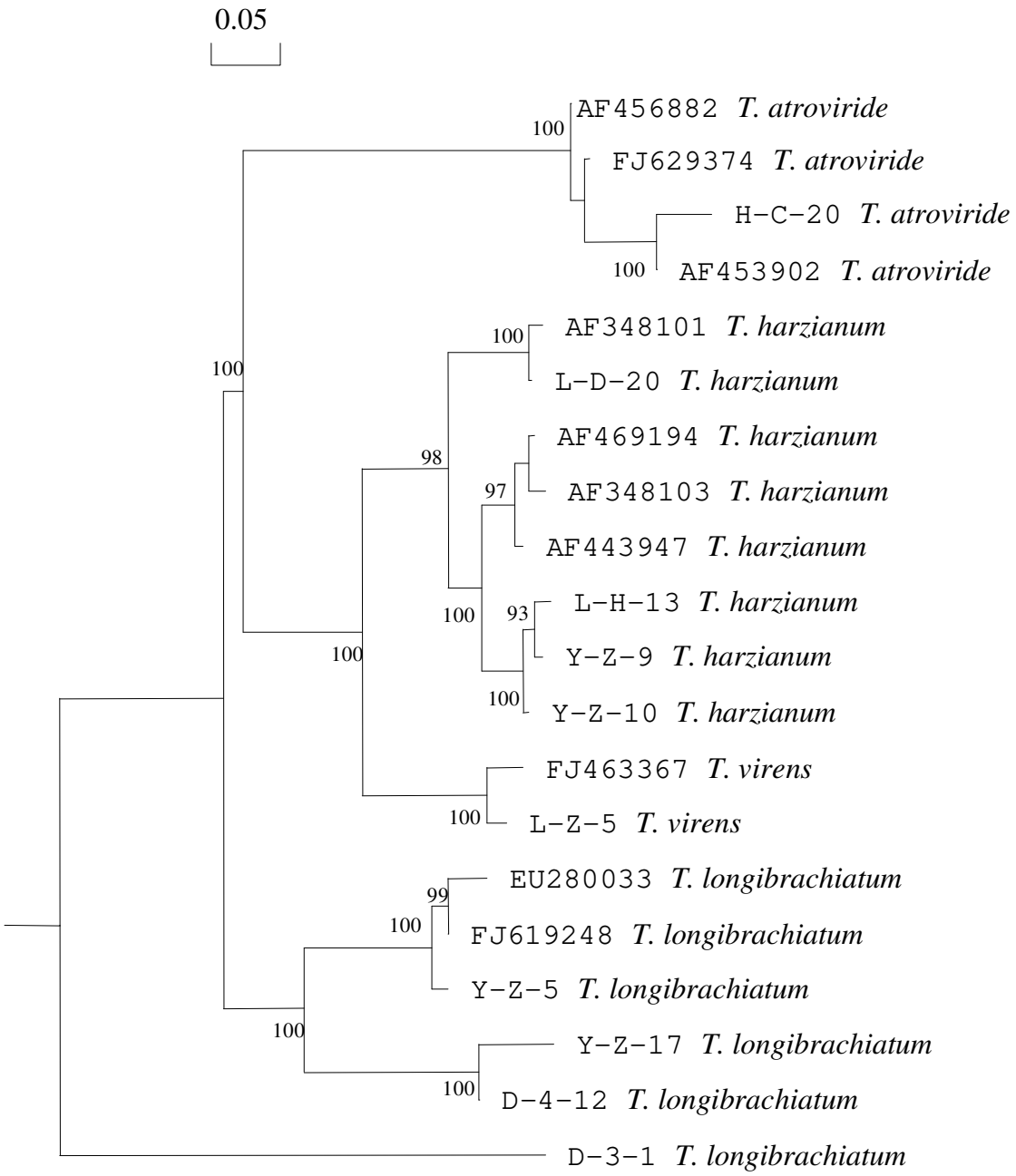

Figure 5. Phylogenetic tree of 10 isolates of *Trichoderma* as inferred by parsimony analysis of ITS1 and ITS2 of *tef1*-sequences for species. There are more than 10 species in this tree.

Maybe, D-3-1 isolate had better antagonism effect on *F. sambucinum*. Further study of the D-3-1 is required.

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