

Article

In Vitro Antagonistic Activity of Indigenous *Trichoderma* Isolates Against *Lasiodiplodia* Isolates Associated with Stem-End Rot of Star Apple (*Chrysophyllum cainito* L.)

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Highlights

What are the main findings?

- Five indigenous *Lasiodiplodia* strains were confirmed as causal agents of stem-end rot disease in star apple fruits.
- *Trichoderma asperellum* T-SA05, *T. yunnanense* T-SA30, and *T. viride* T-SA32 effectively suppressed the growth of selected *Lasiodiplodia* spp. under in vitro conditions.
- The selected *Trichoderma* strains showed antagonistic efficiencies of 49.9–82.4% and produced cell wall-degrading enzymes, including chitinase and β -1,3-glucanase.

What are the implications of the main findings?

- The detection of *L. venezuelensis* and *L. egyptiacae* expands current knowledge of stem-end rot pathogens in star apple.
- Indigenous *Trichoderma* strains show potential as biological control agents for sustainable management of postharvest stem-end rot in star apple.

Abstract

Stem-end rot (SER) is an important postharvest disease that reduces the quality and marketability of star apple (*Chrysophyllum cainito* L.), while biological control offers a more sustainable alternative to chemical management. This study aimed to (i) select fungal strains causing SER disease in star apple and (ii) select and appraise strains of *Trichoderma* spp. fungi as antagonists to the selected fungi causing SER disease under in vitro conditions. Ten fungal isolates were obtained from symptomatic fruits, and 35 *Trichoderma* isolates were recovered from star apple rhizosphere soils. Five rapidly growing fungal isolates were evaluated using a detached-fruit pathogenicity assay, with three fruits per treatment. The five strains with the strongest expression of SER were selected and identified as *Lasiodiplodia theobromae* L-SA01, L-SA03, and L-SA07, *L. venezuelensis* L-SA02, and *L. egyptiacae* L-SA06. Among 25 *Trichoderma* isolates screened for extracellular enzyme activities, T-SA05, T-SA30, and T-SA32 were selected for broad antagonistic activity and were provisionally associated with *T. asperellum*, *T. yunnanense*, and *T. viride*, respectively. At 72 h, these isolates showed antagonistic efficiencies of 55.4–58.9% against L-SA01, 64.9–69.6% against L-SA02, 80.8–82.4% against L-SA03, 58.6–59.3% against L-SA06, and 49.9–54.1% against L-SA07. *L. venezuelensis* and *L. egyptiacae* were the two fungal species first found to



Academic Editor: Huali Xue

Received: 25 June 2026

Revised: 22 July 2026

Accepted: 28 July 2026

Published: 19 August 2026

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cause SER. *T. yunnanense* was recently proven to control the *Lasiodiplodia* spp. fungi that cause SER. The selected isolates also exhibited chitinase and glucanase activities, although their enzyme profiles did not fully correspond to their antagonistic efficiencies. These findings identify indigenous *Trichoderma* isolates for further taxonomic confirmation and evaluation in postharvest fruit assays.

Keywords: antagonistic fungi; biological control; *Chrysophyllum cainito*; *Lasiodiplodia*; postharvest disease; star apple; stem-end rot; *Trichoderma*

1. Introduction

Star apples (*Chrysophyllum cainito* L.) are one of the most popular fruit crops in Vietnam. Can Tho City, Vietnam, possesses more than 1600 ha of farming area for star apples, of which the Phong Dien district accounts for 1075 ha. They have a sweet and favorable taste and can bring about many health benefits for consumers [1]. Recently, star apples have provided high economic value as they are widely consumed domestically and internationally [2]. However, the commercial value of harvested fruits can be substantially reduced by postharvest fungal diseases that impair their external appearance, flesh quality, storage life, and marketability [3,4].

Some pathogenic fungal species reduce the fruit quality of star apples after harvesting, notably *Lasiodiplodia* spp. fungi, which cause stem-end rot (SER) disease in star apples [5–7]. SER is a postharvest disease complex in which decay generally begins near the fruit stem or pedicel and subsequently spreads into the surrounding tissues [8]. Typical symptoms include darkening and wrinkling around the stem end, followed by softening, water-soaked decay, and progressive breakdown of the flesh [9]. SER pathogens may colonize fruit tissues before harvest and remain latent until ripening, senescence, mechanical injury, or unfavorable storage conditions facilitate disease development [10,11]. Because several fungal taxa can produce similar SER symptoms, isolation and characterization of the fungi associated with diseased fruits are necessary before suitable management measures can be developed.

Species of *Lasiodiplodia* are frequently associated with stem-end rot, fruit rot, dieback, cankers, and other diseases of tropical and subtropical crops [12–14]. *Lasiodiplodia theobromae* has previously been reported as a causal agent of SER in star apples [7]. The pathogenicity of *Lasiodiplodia* may involve rapid tissue colonization, secretion of plant cell wall-degrading enzymes, production of secondary metabolites, and responses to environmental conditions that regulate virulence-related genes [15–18]. Nevertheless, the diversity of *Lasiodiplodia* associated with SER of star apples in the Mekong Delta has received limited investigation.

The use of pesticides adversely affects the environment and human health [19]. Thus, a biological approach is considered to be a promising way to control pests, along with being environmentally friendly [20–22]. Among biological methods, fungi of *Trichoderma* spp. are widely used to control plant diseases [23]. Species of *Trichoderma* suppress phytopathogenic fungi through several complementary mechanisms, including competition for space and nutrients, mycoparasitism, production of antifungal metabolites, and secretion of cell wall-degrading enzymes such as chitinases and β -1,3-glucanases [24–26]. *Trichoderma* spp. fungi fight against many plant pathogenic fungi, e.g., *Fusarium* sp., *Rhizoctonia solani*, *Sclerotium rolfsii*, *Botrytis cinerea* and *Aspergillus niger* [24,27]. *Trichoderma* spp. are also known as a biofungicide for crops to resist risks [27]. Nowadays, the application of *Trichoderma* to control pathogenic fungi on crops seems to be a sustainable approach [28–30]. However, the relative contribution of each mechanism varies among isolates and pathogen–antagonist combinations. Therefore,

rapid growth or high activity of an individual hydrolytic enzyme does not necessarily predict the overall antagonistic performance of a *Trichoderma* isolate [31].

Indigenous *Trichoderma* isolates obtained from the crop rhizosphere may be adapted to local environmental conditions and may be candidates for biological management of postharvest pathogens. However, information on indigenous *Trichoderma* associated with star apple orchards and their activity against SER-associated *Lasiodiplodia* isolates is limited. Therefore, this study was conducted with the aims to (i) isolate, select and identify fungal strains that exhibit SER disease in star apples in Can Tho, Vietnam, and (ii) isolate, screen, identify and evaluate strains of *Trichoderma* spp. for their ability to antagonize the selected SER-causing fungi in star apples.

2. Materials and Methods

2.1. Experimental Location and Culture Media

The experiment was carried out from March 2022 to November 2022 in the Edible and Medicinal Mycobiology Laboratory, Faculty of Crop Science, College of Agriculture, and in the Microbiology Laboratory, Department of Microbiology, Food and Biotechnology Institute, Can Tho University.

The solid *Trichoderma*-selective medium (TSM) [32] for isolating fungi of *Trichoderma* was made of NH_4NO_3 (1.00), K_2HPO_4 (0.90), KCl (0.15), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.20), chloramphenicol (0.25) and agar (20.0) (g L^{-1}). The medium for isolating the pathogens was Potato Dextrose Agar (PDA), which was made of (250.0), D-glucose (20.0), chloramphenicol (0.25) and agar (20.0) (g L^{-1}).

2.2. Isolation and Pathogenicity Assessment of Fungi Associated with Stem-End Rot

2.2.1. Fruit Sampling and Preparation

Ten fruit samples with signs of the disease were collected at ten different star apple farms in Truong Khuong A hamlet, Truong Long commune, Phong Dien district, Can Tho city. The signs of SER indicated that the disease was spreading fast to the flesh, causing it to become brown, soft and wet. Then, all of the fruit would become rotten after a few days [33]. The infected fruits were put into nylon bags, labeled, and transported back to the laboratory to isolate pathogens. The samples were washed, surface sterilized with 70% ethanol, washed again with sterilized distilled water, dried with sterile tissue paper, put in an open area and observed for accurate signs of SER disease for one to three days.

2.2.2. Isolation and Morphological Characterization of Fungi

According to Burgess et al. [34], the isolation of pathogenic fungi is based on basic determinations of morphology, symptoms and features of that pathogen on fruits. The fruit samples showed signs of the disease below the stem end, making surrounding tissues dark, wrinkled and soft. A sterile blade was used to collect a disease sample in a V-shape near the stem end at the border between the infected and uninfected tissues; then, the sample was put into a Petri dish containing PDA medium. Subsequently, the dishes were incubated for 5 days at 28 °C in an incubator, and inoculations were carried out until the fungi were pure and uniform in color, colony surface and hyphae shape. After that, the morphological characteristics of the colonies were observed.

The shapes of mycelia and the shapes and colors of hyphae and spores were observed by a stereoscope. Pathogenic fungal strains were inoculated on PDA medium and measured for growth after 24, 48, 72 and 96 h of inoculation.

2.2.3. Detached-Fruit Pathogenicity Assay

Each of the five pathogenic fungal strains was inoculated onto the star apple fruits through an artificial wound, after the fruits' surfaces were sterilized and the fungal density was adjusted to 1×10^6 CFU/mL. The experiment was completely randomized with 6 treatments, including the five pathogenic fungi strains and the control treatment, with three replications representing three star apple fruits. The wounds were made by a needle at 3 spots on the fruit stalk and 5 spots on the fruit peel; then, 1 μ L of the fungal suspension was injected into each wound on the star apple fruits. The control treatment was applied with distilled water. The infected wounds and the ratios of rotten fruits were investigated.

2.3. Isolation and Screening of Indigenous *Trichoderma*

2.3.1. Soil Sampling and Isolation of *Trichoderma*

Thirty-five rhizosphere samples were collected from 35 different star apple farms in Truong Khuong A hamlet. The soil samples were collected 1.0–1.2 m from the trunk of a healthy star apple tree at a depth of 5–20 cm (100 g per sample); they were then transferred to a clean bag, labeled and transported back to the laboratory. The soil samples were stored at 4 °C and cleaned of organic residues before *Trichoderma* spp. isolation.

The isolation and collection of the fungal source were performed according to the method of Kumar et al. [35] in TSM medium. In particular, the soil samples were mixed with sterilized distilled water at a ratio of 1.0 g soil and 99 mL sterilized distilled water, shaken for 30 min and left for sedimentation for 24 h at room temperature (30–32 °C). Subsequently, a 200 μ L liquid soil sample was added to the TSM, which was sterilized at 121 °C for 20 min. After 48 h, the isolated samples were checked and observed for colony selection. After that, detachment and purification were conducted on PDA medium. The strains were named after the *Trichoderma* (T) genus, the star apple (SA), and the order number of sampling positions from 01 to 35, i.e., from T-SA01 to T-SA35.

2.3.2. Morphological Characterization and Mycelial Growth

The colors of mycelia and types of hyphae and spores were observed under a light microscope at 40 $\times$ to detect *Trichoderma* spp. Pure samples were stored in Petri dishes with PDA medium at 4 °C and in a 2 mL cryotube (20% glycerol).

Fungal strains were cultured in dishes with PDA medium at 28 ± 2 °C for a week; their colony diameters were measured at 24, 48, 72 and 96 h after incubation, with three replications. Strains with good growth were used for evaluation in the following experiments.

2.3.3. Extracellular Enzyme Assays

A total of 25 *Trichoderma* spp. strains with excellent growth rates were assessed for their enzyme-secreting capacity. All of the experiments followed a completely randomized design with three replications. The *Trichoderma* spp. strains cultured for 7 days on PDA media were propagated in 50 mL tubes containing 10 mL of liquid TSM, with a density of 10^7 spores mL⁻¹ under room conditions and shaken at 120 rpm for 5 days. After that, 2 mL of the liquid was collected and centrifuged at 5000 rpm for 20 min to remove biomass, and the supernatant was used as a source of raw extracellular enzymes. Therein, the liquid TSM was supplied with 0.5% chitin to evaluate the ability to secrete chitinase, exo- β -1,3-glucanase, and endo- β -1,3-glucanase, or with 0.5% CMC—carboxymethyl cellulose to assess the activity of cellulase. Fungal strains of *Trichoderma* spp. with a potential capability of producing chitinase, cellulase, exo- β -1,3-glucanase, and endo- β -1,3-glucanase were chosen to antagonize *Lasiodiplodia* spp. fungi.

The activity of chitinase is estimated based on the amount of reduced sugar produced via an indirect reaction with the Nelson–Somogyi reagent, in which a brownish-red color is

formed when heated, and when reacted with the Arsenomolybdate reagent, the color turns sky blue. The obtained liquid was measured at a 520 nm wavelength in a spectrophotometer. The chitinase activity was measured by adding 1 mL of the enzyme and reacting with 1 mL of chitinase at 40 °C for 60 min, after which the enzyme activity (UI mL⁻¹) was calculated [36].

From each fungal strain, 20 µL of liquid extracellular enzymes was transferred into 3 wells (0.5 cm) in the TSM, with sterilized distilled water as the control well, and incubated at 37 °C for 5 days. Then, Lugol dying was carried out by submerging the Lugol solution on the surface of the media. After 3–5 min, iodine started to combine with cellulose in the matrix of the medium, creating a dark blue complex; when there was no combination, cellulose was hydrolyzed, resulting in a bright zone around the well with the extracellular enzymes, i.e., a clear zone appeared surrounding a well with enzymes [37].

The activity of endo-β-1,3-glucanase was determined by the amount of sugar reduced after reacting 1 mL of the enzyme with 1 mL CMC at 40 °C for 60 min. For the activity of exo-β-1,3-glucanase, CMC was replaced with cellulose powder (UI mL⁻¹) [38].

2.4. In Vitro Dual-Culture Antagonism Assay

The experiment was designed with complete randomization and three replications. The fungal strains of *Trichoderma* spp. and *Lasiodiplodia* spp. were raised for 7 days on PDA media. The *Trichoderma* spp. and *Lasiodiplodia* spp. were cultured on the same Petri dishes, with a distance between the two colony centers of 6 cm on the two opposite sides of a Petri dish (90 mm in diameter) at 28 ± 2 °C [39]. The radii of the colonies of *Lasiodiplodia* spp. were measured, and antagonistic efficiencies (AEs) were determined at 24, 48, 72 and 96 h after inoculation.

$$AE(\%) = \frac{(Control - T)}{Control} \times 100$$

where Control is the growth radius of the pathogen in the control treatment and T is the growth radius of the *Lasiodiplodia* spp. in the treatment with the *Trichoderma* spp.

Evaluation of AEs: An AE value not lower than 60% means high resistance, from 40% to 59% means moderate resistance, from 20% to 39% means weak resistance and not higher than 19% means no resistance.

2.5. ITS Sequencing and Phylogenetic Placement

DNA extractions of the SER pathogens L-SA01, L-SA02, L-SA03, L-SA06 and L-SA07 and the *Trichoderma* spp. T-SA05, T-SA30 and T-SA32 were performed at 7 days after inoculation on PDA media [40]. The fungal spores on the PDA media were transferred into 2.2 mL Eppendorf tubes, shaken, incubated at room temperature for 10 min and centrifuged at 13,000 rpm for 5 min to collect extracts, which were then transferred into new Eppendorf tubes, rinsed with 500 µL of 70% ethanol, centrifuged at 13,000 rpm for 5 min and vacuum-dried. Subsequently, DNA samples were dissolved in 100 µL TE 0.1×. Then, PCR reactions were started with a primer pair: ITS 1: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS 4: 5'-TCCTCCGCTTATTGATATGC-3' [41]. The PCR reactions were carried out in a volume of 50 µL and followed a thermal protocol: denaturation (95 °C for 5 min) and a 30-cycle procedure (denaturation at 95 °C for 90 s, annealing at 52 °C for 60 s and extension at 72 °C for 90 s). The amplicons were purified and sequenced by an automatic sequencing method. Based on the sequencing results, comparisons were made with the GenBank database by the Basic Local Alignment Search Tool (BLAST, version 2.17.0), of the National Center for Biotechnology Information (NCBI). The fungal sequences were used to construct a phylogenetic tree in MEGA 6.0 software.

2.6. Statistical Analysis

Data were subjected to one-way analysis of variance. When significant treatment effects were detected, means were separated using Duncan's multiple range test at $p < 0.05$. Each laboratory assay included three independent replicates. Statistical analyses were performed separately for each observation time and each pathogen–antagonist combination.

3. Results

3.1. Isolation and Pathogenicity of Fungi Associated with Stem-End Rot

3.1.1. Isolation and Morphological Characteristics of the Pathogens

Using 10 fruit samples with signs of disease from 35 star apple farms, the isolation and purification resulted in 10 fungal strains that caused SER disease on PDA media. The pathogenic hyphae grew fast and had uneven round shapes on the PDA media at 8 DAI. Colonies were initially white and generally developed light- to dark-green pigmentation after 48 h of incubation. Structures of hyphae were diverse, fluffy and well spread. All of the 10 strains possessed white colonies, which turned dark brown due to spores. At 8 DAI, some of them began to show fungal nests (Figure 1).

3.1.2. Mycelial Growth of the Pathogens

The growth rates of mycelia after 24, 48, 72 and 96 h of inoculation differed at 5% significance between strains. In particular, the fastest growth rates were recorded for the L-SA07 strain at 24 h (0.62 mm h^{-1}), the L-SA01 strain at 48 h (0.61 mm h^{-1}), the L-SA06 strain at 72 h (1.13 mm h^{-1}) and the L-SA01, L-SA02 and L-SA03 strains at 96 h (1.09 mm h^{-1}) (Table 1). Therefore, the L-SA01, L-SA02, L-SA03, L-SA06 and L-SA07 strains were chosen to proceed to the next experiment.

3.1.3. Detached-Fruit Pathogenicity

All of the fungal strains were responsible for the disease based on the disease's symptoms and rotten fruits observed after 2 days and 4 days of inoculation, respectively. Moreover, the rotten fruit ratio was 100% on day 4 (Figure 2).

Table 1. Growth rate of fungi causing stem-end rot disease in star apples on the PDA medium.

Isolate	Growth Rate of Mycelia (mm/h)			
	24 h	48 h	72 h	96 h
L-SA01	0.58 ^d	0.61 ^a	1.01 ^c	1.09 ^a
L-SA02	0.61 ^b	0.55 ^{cd}	1.03 ^c	1.09 ^a
L-SA03	0.58 ^d	0.56 ^c	1.01 ^c	1.09 ^a
L-SA04	0.53 ^e	0.46 ^f	0.93 ^{de}	0.98 ^b
L-SA05	0.46 ^g	0.50 ^e	0.94 ^{de}	0.98 ^b
L-SA06	0.60 ^c	0.53 ^d	1.13 ^a	1.01 ^b
L-SA07	0.62 ^a	0.58 ^b	1.06 ^b	1.01 ^b
L-SA08	0.52 ^f	0.44 ^g	0.96 ^d	0.85 ^d
L-SA09	0.53 ^e	0.42 ^g	0.23 ^e	0.93 ^c
L-SA10	0.51 ^f	0.46 ^f	0.86 ^f	0.97 ^b
F	*	*	*	*
CV (%)	1.29	1.84	5.74	2.17

Note: In the same column, the same letters mean an insignificant difference based on the Duncan post hoc test. (*) different at 5% significance.

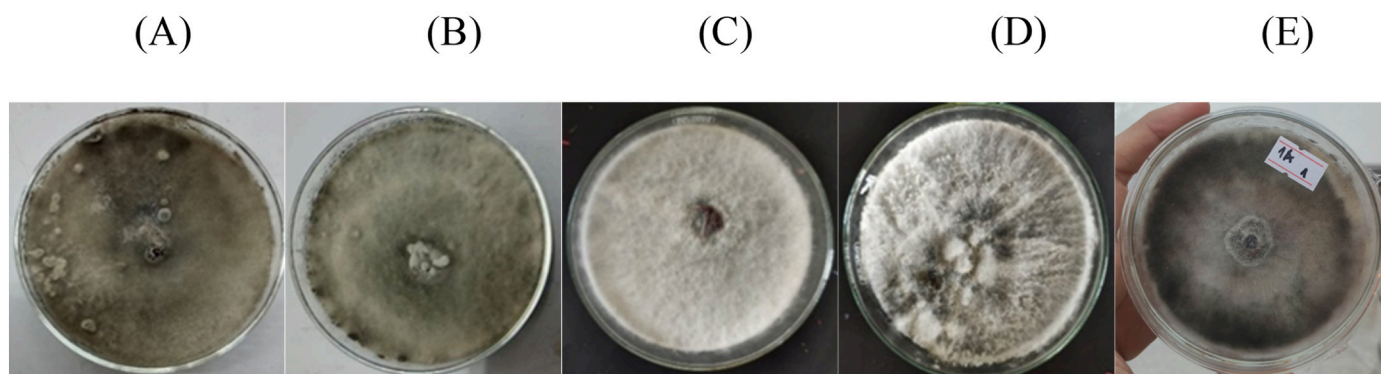


Figure 1. Mycelial morphology of *Lasiodiplodia* spp. causing stem-end rot disease in star apples: (A) L-SA01, (B) L-SA02, (C) L-SA03, (D) L-SA06 and (E) L-SA07 strains after 8 days of inoculation on PDA media.

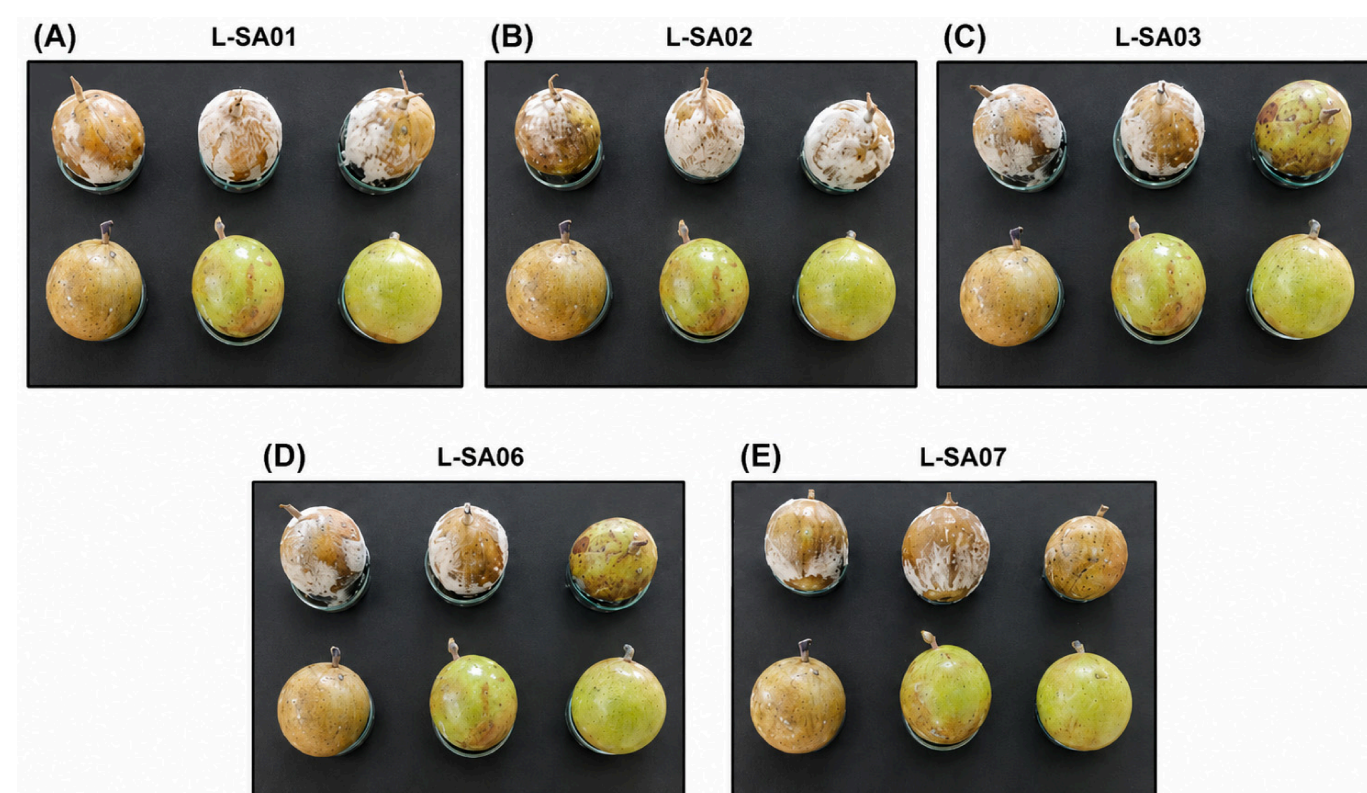


Figure 2. Symptoms of stem-end rot in detached star apple fruits at 4 days after inoculation with the selected fungal isolates: (A) L-SA01, (B) L-SA02, (C) L-SA03, (D) L-SA06, and (E) L-SA07. In each panel, the upper row shows inoculated fruits, whereas the lower row shows control fruits treated with sterile distilled water. Each treatment consisted of three fruits. Browning, softening, and decay were observed in the inoculated fruits, while no disease symptoms developed in the controls.

3.2. ITS-Based Phylogenetic Placement of Selected *Lasiodiplodia* Isolates

After sequencing genes in the ITS region, the L-SA01, L-SA03 and L-SA07 strains were identified as *Lasiodiplodia theobromae* with the corresponding accession numbers of OP984145, OP984147 and OP984149 in GenBank. The L-SA02 and L-SA06 strains were identified as *L. venezuelensis* and *L. egyptiacae*, with the accession numbers of OP984146 and OP984148, with 100% similarities (Figure 3).

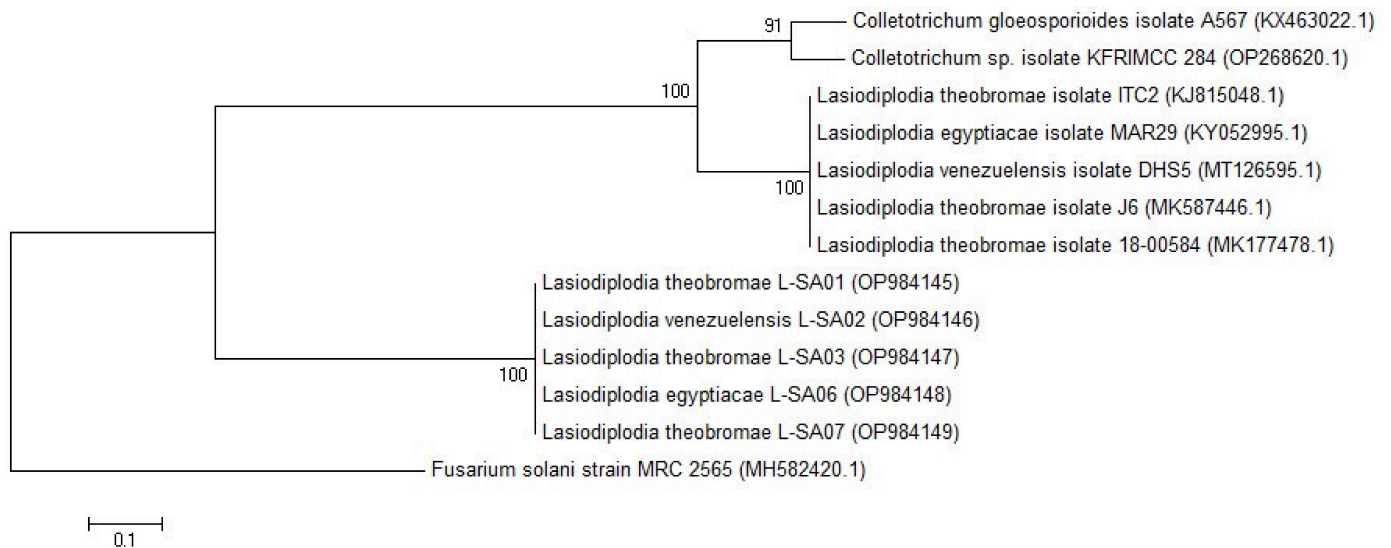


Figure 3. Maximum likelihood tree of *Lasiodiplodia* spp. from star apple fruits and other *Colletotrichum* spp. based on the analysis of the internal transcribed spacer region, with *Fusarium solani* MRC 2565 as an outgroup. Bootstrap values are indicated at the nodes (1000 replicates). GenBank accession numbers are given in parentheses. The scale bar represents the number of substitutions per site.

3.3. Indigenous *Trichoderma* to Control *Lasiodiplodia* spp.

3.3.1. Isolation and Morphology of *Trichoderma* spp. Strains

A total of 35 strains of *Trichoderma* spp. were isolated in the rhizosphere from 35 star apple farms (Figure 4). The isolated fungi introduced white colonies that turned green after 48 h of inoculation, whose hyphae grew fast, were fluffy, and formed a concentric circle in each dish. The strains formed long and thick conidiophores with short and thick branches. The peak of each branch was shaped like a short bottle. There was evidence of oval conidia in low quantities.

3.3.2. Mycelial Growth of *Trichoderma* spp. Strains

Growth rates of the *Trichoderma* spp. strains were different at 5% after 24, 48, 72 and 96 h of inoculation. After 24 h, high-growing colony diameters included the *Trichoderma* spp. T-SA14, T-SA16, T-SA19, T-SA26, T-SA30 and T-SA32 strains, whose diameters fluctuated roughly 33.0–36.2 mm day^{−1}. From 48 to 72 h, the T-SA16, T-SA19, T-SA25 and T-SA26 strains had superior growth rates, at 47.7–67.7, 47.3–67.3, 49.3–68.4 and 48.3–68.3 mm, respectively. Up to 96 h, because the strains had covered the entire surface of the dishes, the diameters of many strains were equivalent and valued at approximately 75.9–90.0 mm (Table 2). Twenty-five fungal strains with a fine growth rate were selected for the next experiment.

3.3.3. Extracellular Enzyme Activities

Chitinase activity: The activities of chitinase varied at 5% significance between the fungal strains. Three *Trichoderma* spp. strains with high enzymatic activities were the T-SA32 > T-SA25 ~ T-SA05 strains, corresponding to 0.047 > 0.037 ~ 0.037 UI mL^{−1}. Moreover, strains with chitinase activities lower than 0.02 UI mL^{−1} included the T-SA01, T-SA02, T-SA04, T-SA10, T-SA11, T-SA12, T-SA15, T-SA18, T-SA20, T-SA24 and T-SA33 strains, accounting for 44% of the total strains, while the higher enzymatic activity group accounted for 56% (Table 3).

β-1,3-glucanase activity: The strains with high enzymatic activities of endo-β-1,3-glucanase were the T-SA03, T-SA05 and T-SA14 strains, with corresponding activities

of 0.0530 ~ 0.0517 ~ 0.0457 UI mL⁻¹. However, the *Trichoderma* spp. strains possessing low activities of endo- β -1,3-glucanase (<0.010 UI mL⁻¹) included the T-SA02, T-SA06, T-SA11, T-SA12 and T-SA23 strains. The T-SA05 strain resulted in the highest exo- β -1,3-glucanase activity (0.0520 UI mL⁻¹) (Table 3).

Cellulase activity: The qualitative result of cellulase activities of *Trichoderma* was roughly 0.90–2.50 cm (Table 3). To be more specific, the T-SA02, T-SA05, T-SA06, T-SA13, T-SA15, T-SA19, T-SA20, T-SA23 and T-SA24 strains had large clear zones, whose diameters ranged from 1.90 to 2.50 cm (Table 3).

Table 2. Growth rate of *Trichoderma* spp. on PDA medium.

Isolate	Growth Rate of Fungi (mm)			
	24 h	48 h	72 h	96 h
T-SA01	24.4 ^{hi}	40.6 ^{g-j}	60.6 ^{g-j}	88.0 ^{ab}
T-SA02	24.1 ^{hi}	41.6 ^{f-i}	61.6 ^{f-i}	88.3 ^{ab}
T-SA03	32.3 ^{bc}	41.8 ^{f-i}	61.5 ^{f-i}	90.0 ^a
T-SA04	31.3 ^c	43.9 ^{def}	63.9 ^{def}	89.7 ^a
T-SA05	32.0 ^{bc}	43.8 ^{def}	63.8 ^{def}	90.0 ^a
T-SA06	23.7 ^{hi}	34.7 ^o	54.7 ^o	88.7 ^{ab}
T-SA07	23.5 ⁱ	39.1 ^{i-l}	59.1 ^{i-m}	79.1 ^{de}
T-SA08	31.9 ^{bc}	42.7 ^{efg}	62.7 ^{efg}	90.0 ^a
T-SA09	25.5 ^{e-i}	36.2 ^{mno}	56.8 ^{l-o}	76.8 ^{de}
T-SA10	26.5 ^{d-h}	42.1 ^{e-h}	62.1 ^{e-h}	88.9 ^{ab}
T-SA11	24.3 ^{hi}	36.9 ^{l-o}	56.9 ^{k-o}	88.8 ^{ab}
T-SA12	25.2 ^{f-i}	36.2 ^{mno}	56.2 ^{mno}	88.9 ^{ab}
T-SA13	28.2 ^{de}	37.8 ^{k-n}	57.8 ^{j-n}	85.9 ^{bc}
T-SA14	36.2 ^a	45.6 ^{bcd}	65.6 ^{bcd}	89.7 ^a
T-SA15	24.8 ^{ghi}	38.1 ^{k-n}	58.1 ^{j-n}	86.7 ^{abc}
T-SA16	34.1 ^{ab}	47.7 ^{ab}	67.7 ^{ab}	90.0 ^a
T-SA17	25.6 ^{e-i}	38.3 ^{k-n}	58.3 ^{j-n}	78.3 ^{de}
T-SA18	25.3 ^{f-i}	41.8 ^{f-i}	61.8 ^{f-i}	83.5 ^c
T-SA19	34.2 ^{ab}	47.3 ^{abc}	67.3 ^{abc}	90.0 ^a
T-SA20	27.2 ^{d-g}	37.4 ^{l-o}	57.4 ^{k-o}	87.9 ^{ab}
T-SA21	27.2 ^{d-g}	38.4 ^{k-n}	58.4 ^{j-n}	78.4 ^{de}
T-SA22	25.1 ^{f-i}	38.0 ^{k-n}	58.0 ^{j-n}	78.0 ^{de}
T-SA23	26.0 ^{d-i}	36.2 ^{mno}	56.2 ^{mno}	86.8 ^{abc}
T-SA24	23.7 ^{hi}	39.9 ^{g-k}	59.9 ^{h-k}	88.9 ^{ab}
T-SA25	32.7 ^{bc}	49.3 ^a	68.4 ^a	90.0 ^a
T-SA26	34.3 ^{ab}	48.3 ^{ab}	68.3 ^a	89.8 ^a
T-SA27	28.5 ^d	38.3 ^{k-n}	58.3 ^{j-n}	77.9 ^{de}
T-SA28	23.3 ⁱ	37.9 ^{k-n}	57.9 ^{j-n}	77.9 ^{de}
T-SA29	25.8 ^{e-i}	35.6 ^{no}	55.6 ^{no}	75.6 ^e
T-SA30	35.8 ^a	44.8 ^{cde}	64.8 ^{cde}	90.0 ^a
T-SA31	24.5 ^{ghi}	37.4 ^{l-o}	57.4 ^{k-o}	87.7 ^{ab}
T-SA32	33.9 ^{ab}	44.0 ^{def}	64.0 ^{def}	89.7 ^a
T-SA33	27.6 ^{def}	37.1 ^{l-o}	57.1 ^{k-o}	87.3 ^{ab}
T-SA34	24.3 ^{hi}	35.9 ^{no}	55.9 ^{no}	75.9 ^e
T-SA35	24.1 ^{hi}	39.5 ^{h-k}	59.5 ^{h-l}	79.5 ^d
F	*	*	*	*
CV (%)	3.88	2.94	1.93	1.68

Note: In the same column, the same letters mean an insignificant difference based on the Duncan post hoc test. (*) different at 5% significance.

Table 3. Enzyme activity of the selected *Trichoderma* spp.

Isolates	Chitinase (UI/mL)	Endo- β -1,3-glucanase (UI/mL)	Exo- β -1,3-glucanase (UI/mL)	Cellulase (cm)
T-SA01	0.009 j	0.0290 de	0.0120 ijk	1.67 c-g
T-SA02	0.019 e-j	0.0090 lm	0.0150 h-k	1.93 a-e
T-SA03	0.033 bc	0.0530 a	0.0403 b	1.57 d-g
T-SA04	0.015 f-j	0.0227 e-h	0.0250 d-g	1.50 d-g
T-SA05	0.037 b	0.0517 a	0.0520 a	2.07 a-d
T-SA06	0.026 c-f	0.0097 lm	0.0107 jk	1.93 a-e
T-SA08	0.029 b-e	0.0193 f-k	0.0210 e-i	1.23 efg
T-SA10	0.013 g-j	0.0223 e-h	0.0090 k	1.53 efg
T-SA11	0.014 g-j	0.0027 m	0.0257 def	1.40 d-g
T-SA12	0.009 j	0.0087 lm	0.0267 def	1.67 c-g
T-SA13	0.022 c-h	0.0220 e-h	0.0330 bcd	2.50 ab
T-SA14	0.024 b-f	0.0457 ab	0.0193 f-j	1.13 fg
T-SA15	0.012 hij	0.0253 d-g	0.0243 d-h	1.90 a-f
T-SA16	0.020 d-i	0.0177 g-l	0.0270 def	1.77 b-f
T-SA18	0.015 f-j	0.0213 e-h	0.0213 e-i	1.13 fg
T-SA19	0.029 bcde	0.0153 hkl	0.0400 b	2.00 a-e
T-SA20	0.010 ij	0.0260 d-g	0.0097 jk	2.13 a-d
T-SA23	0.030 bcd	0.0060 m	0.0300 cde	2.17 a-d
T-SA24	0.019 d-j	0.0110 klm	0.0377 bc	2.37 abc
T-SA25	0.037 b	0.0340 cd	0.0300 cde	0.90 g
T-SA26	0.032 bc	0.0387 bc	0.0160 g-k	2.63 a
T-SA30	0.027 b-e	0.0187 ghk	0.0383 bc	1.47 d-g
T-SA31	0.022 c-h	0.0160 hkl	0.0117 ijk	1.53 d-g
T-SA32	0.047 a	0.0280 def	0.0263 def	1.53 d-g
T-SA33	0.019 d-j	0.0257 d-g	0.0153 h-k	1.60 c-g
F	*	*	*	*
CV (%)	19.0	16.3	15.5	17.3

Note: In the same column, the same letters mean an insignificant difference based on the Duncan post hoc test. (*) different at 5% significance.

3.4. In Vitro Antagonism Against SER-Associated Isolates

The AEs of *Trichoderma* spp. against the *Lasiodiplodia theobromae* L-SA01, *L. venezuelensis* L-SA02, *L. theobromae* L-SA03, *L. egyptiacae* L-SA06 and *L. theobromae* L-SA07 strains differed at 5% significance after 48 and 72 h of inoculation (Figure 5; Tables 4–8). In particular, after 72 h, the antagonism capacities of the *Trichoderma* spp. strains against the *Lasiodiplodia* spp. strains were 31.0–58.9, 42.1–69.6, 70.5–82.4, 44.7–59.3 and 17.8–54.1% (Tables 4–8). The three *Trichoderma* spp. strains that best antagonized *L. theobromae* L-SA01 were the T-SA05, T-SA30 and T-SA32 strains, with AEs of approximately 55.4–58.9% (Table 4).

Likewise, for the *L. venezuelensis* L-SA02 pathogenic strain, the T-SA05, T-SA08, T-SA14, T-SA18, T-SA25, T-SA26, T-SA30 and T-SA32 antagonistic strains had the highest results (61.9–69.6%) (Table 5); for the *L. theobromae* L-SA03 strain, the T-SA05, T-SA30 and T-SA32 strains had the highest results (80.8–82.4%) (Table 6); for the *L. egyptiacae* L-SA06 strains, the T-SA05, T-SA14, T-SA19, T-SA30 and T-SA32 strains had the highest results (57.8–59.3%) (Table 7); and for the *L. theobromae* L-SA07 strain, the T-SA05, T-SA30 and T-SA32 strains had the highest results (49.9–54.1%) (Table 8). In short, the three *Trichoderma* spp. T-SA05, T-SA30 and T-SA32 strains were selected as the strongest in inhibiting the growth of the *Lasiodiplodia* spp. L-SA01, L-SA02, L-SA03, L-SA06 and L-SA07 fungi.

3.5. ITS-Based Phylogenetic Placement of Selected *Trichoderma* Isolates

Based on their broad antagonistic activities, T-SA05, T-SA30, and T-SA32 were selected for ITS sequence analysis. At a similarity of 99%, the T-SA05, T-SA30 and T-SA32 strains were identified as *Trichoderma asperellum* T-SA05 (OP984142), *T. viride* T-SA32 (OP984144) and *T. yunnanense* T-SA30 (OP984143) (Figure 6).

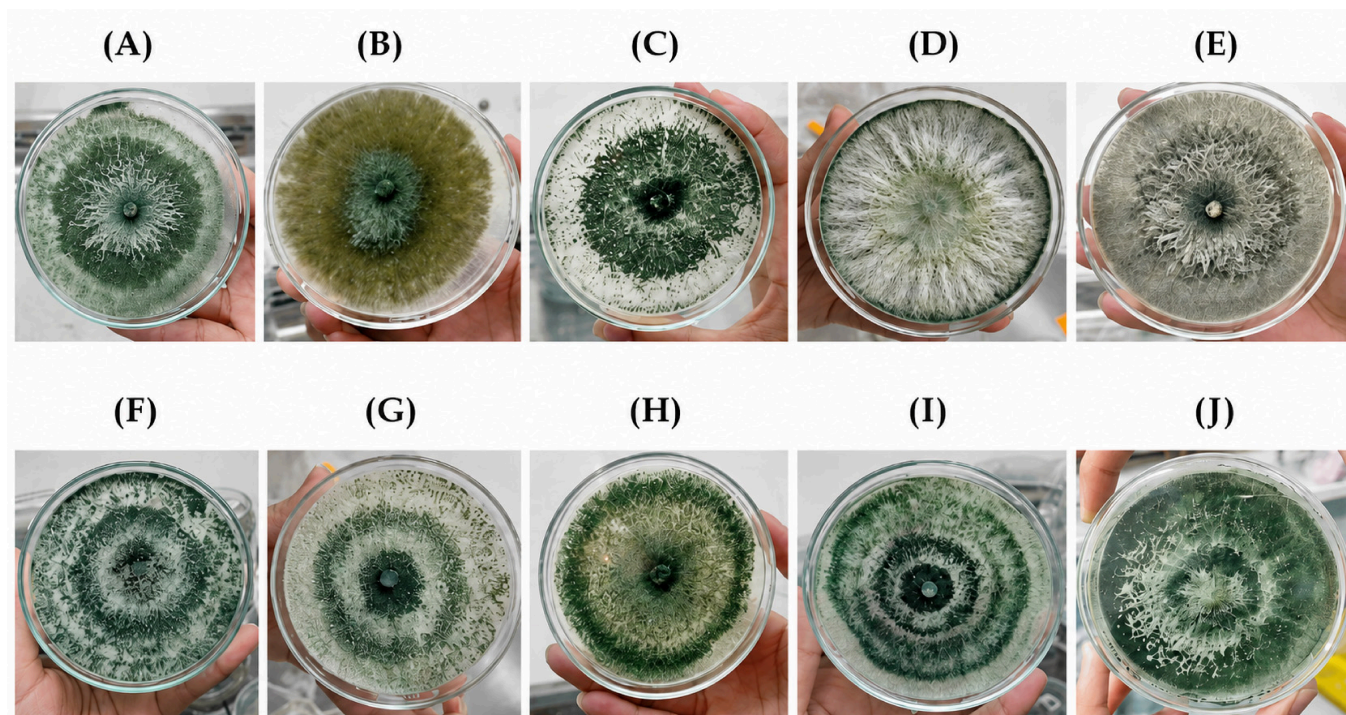


Figure 4. Mycelial morphology of the *Trichoderma* spp. (A) T-SA05, (B) T-SA07, (C) T-SA08, (D) T-SA12, (E) T-SA16, (F) T-SA18, (G) T-SA25, (H) T-SA30, (I) T-SA31 and (J) T-SA32 strains after 8 days of inoculation on PDA media.

Table 4. Antagonistic efficiency of *Trichoderma* spp. against the *Lasiodiplodia theobromae* L-SA01 strain causing stem-end rot disease in star apples.

Isolate	Antagonistic Efficiency (%)		Antagonistic Diameter Against the <i>Lasiodiplodia theobromae</i> L-SA01 Strain (cm)	
	48 h	72 h	48 h	72 h
T-SA03	13.9 ^c	33.0 ^e	0.40 ^c ± 0.24	1.76 ^e ± 0.23
T-SA05	26.9 ^a	55.4 ^b	0.76 ^a ± 0.14	2.95 ^{ab} ± 0.03
T-SA08	8.60 ^e	41.9 ^d	0.24 ^{de} ± 0.01	2.23 ^d ± 0.24
T-SA14	11.2 ^d	47.4 ^c	0.32 ^{cd} ± 0.07	2.53 ^c ± 0.09
T-SA18	4.82 ^e	52.3 ^c	0.14 ^e ± 0.03	2.79 ^{bc} ± 0.13
T-SA19	18.9 ^b	31.0 ^e	0.54 ^b ± 0.06	1.65 ^e ± 0.20
T-SA25	12.1 ^d	51.7 ^c	0.34 ^{cd} ± 0.04	2.79 ^{bc} ± 0.17
T-SA26	18.7 ^b	41.2 ^d	0.24 ^{de} ± 0.04	2.19 ^d ± 0.20
T-SA30	19.2 ^b	58.9 ^a	0.54 ^b ± 0.05	3.14 ^a ± 0.10
T-SA32	29.8 ^a	58.8 ^a	0.84 ^a ± 0.06	3.13 ^a ± 0.17
F	*	*	*	*
CV (%)	15.3	6.14	15.2	6.03

Note: Numbers followed by different letters in the same column are different at 5% significance based on Duncan post hoc test. (*) different at 5% significance.

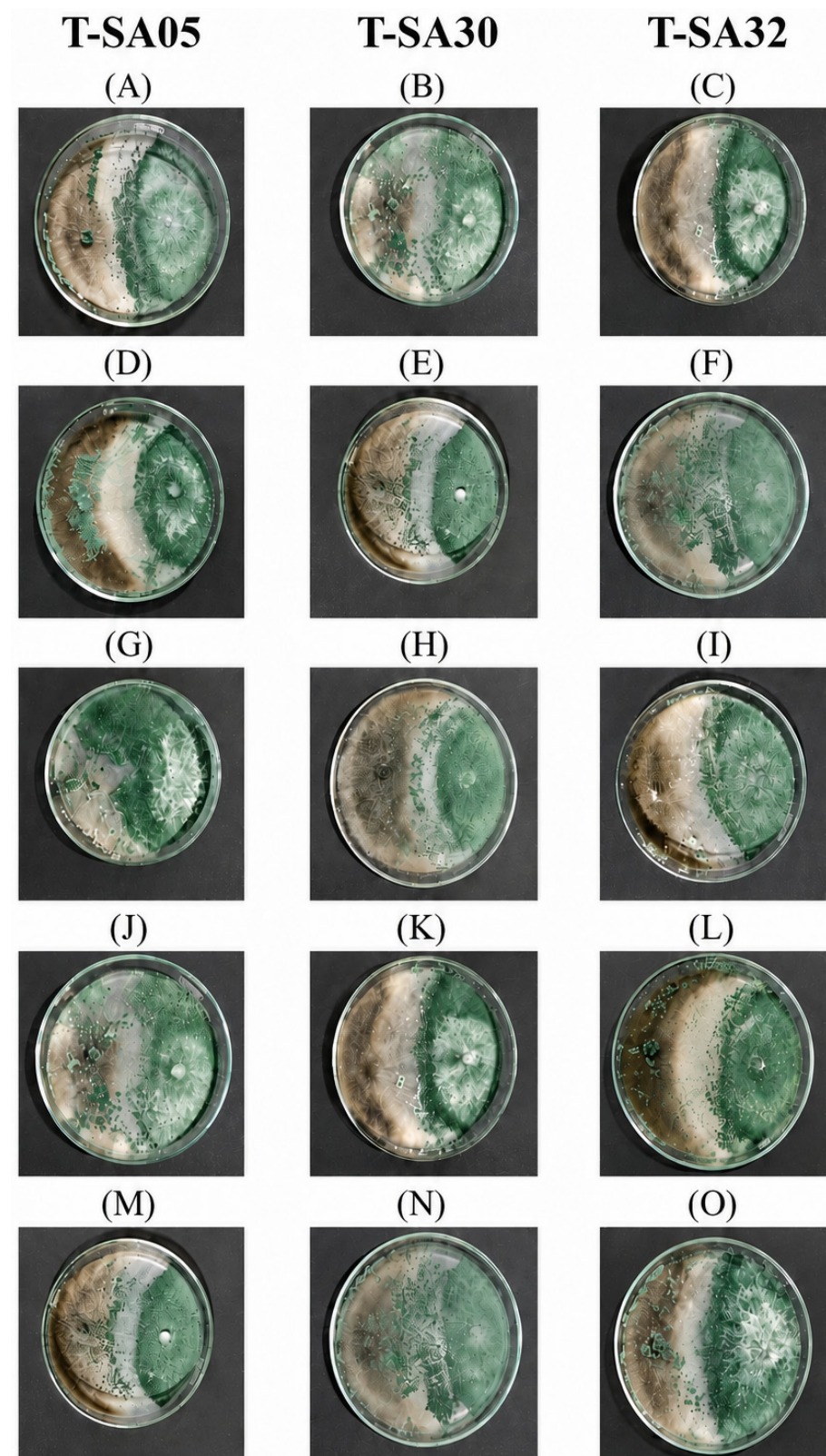


Figure 5. Antagonistic capacity of the *Trichoderma* spp. T-SA05, T-SA30 and T-SA32 strains against *Lasiodiplodia theobromae* L-SA01 (A–C), L-SA02 (D–F), L-SA03 (G–I), L-SA06 (J–L) and L-SA07 (M–O) on PDA media after 4 days of inoculation.

Table 5. Antagonistic efficiency of *Trichoderma* spp. against the *Lasiodiplodia venezuelensis* L-SA02 strain causing stem-end rot disease in star apples.

Isolate	Antagonistic Efficiency (%)		Antagonistic Diameter Against the <i>Lasiodiplodia venezuelensis</i> L-SA02 Strain (cm)	
	48 h	72 h	48 h	72 h
T-SA03	9.85 ^{de}	46.5 ^b	0.27 ^{de} ± 0.04	2.52 ^b ± 0.16
T-SA05	20.3 ^b	69.6 ^a	0.56 ^{ab} ± 0.02	3.77 ^a ± 0.04
T-SA08	10.1 ^e	61.9 ^a	0.28 ^{de} ± 0.05	3.35 ^a ± 0.30
T-SA14	6.97 ^e	61.9 ^a	0.19 ^e ± 0.01	3.35 ^a ± 0.21
T-SA18	12.0 ^d	65.9 ^a	0.33 ^d ± 0.04	3.57 ^a ± 0.06
T-SA19	14.1 ^{cd}	42.1 ^b	0.39 ^{cd} ± 0.14	2.29 ^b ± 0.83
T-SA25	19.9 ^{ab}	60.6 ^a	0.55 ^{ab} ± 0.06	3.29 ^a ± 0.43
T-SA26	17.3 ^{bc}	58.8 ^a	0.48 ^{bc} ± 0.01	3.19 ^a ± 0.21
T-SA30	24.2 ^a	66.8 ^a	0.67 ^a ± 0.19	3.62 ^a ± 0.02
T-SA32	23.3 ^a	64.9 ^a	0.65 ^a ± 0.14	3.52 ^a ± 0.02
F	*	*	*	*
CV (%)	16.9	10.0	17.6	10.0

Note: Numbers followed by different letters in the same column are different at 5% significance based on the Duncan post hoc test. (*) different at 5% significance.

Table 6. Antagonistic efficiency of *Trichoderma* spp. against the *Lasiodiplodia theobromae* L-SA03 strain causing stem-end rot disease in star apples.

Isolate	Antagonistic Efficiency (%)		Antagonistic Diameter Against the <i>Lasiodiplodia theobromae</i> L-SA03 Strain (cm)	
	48 h	72 h	48 h	72 h
T-SA03	17.7 ^{bc}	71.0 ^{de}	0.49 ^{bc} ± 0.06	2.35 ^{de} ± 0.05
T-SA05	16.6 ^c	80.8 ^a	0.46 ^c ± 0.01	3.45 ^a ± 0.12
T-SA08	17.0 ^c	74.2 ^{bc}	0.47 ^c ± 0.01	2.70 ^{bc} ± 0.01
T-SA14	17.7 ^{bc}	75.6 ^{bc}	0.49 ^{bc} ± 0.03	2.99 ^b ± 0.26
T-SA18	21.1 ^a	73.2 ^{cd}	0.58 ^a ± 0.05	2.59 ^{cd} ± 0.29
T-SA19	19.7 ^{ab}	70.5 ^e	0.54 ^b ± 0.03	2.29 ^e ± 0.19
T-SA25	17.0 ^c	76.8 ^b	0.47 ^c ± 0.01	2.99 ^b ± 0.10
T-SA26	16.3 ^c	73.8 ^c	0.45 ^c ± 0.02	2.66 ^c ± 0.21
T-SA30	20.9 ^a	81.3 ^a	0.57 ^a ± 0.02	3.50 ^a ± 0.02
T-SA32	21.4 ^a	82.4 ^a	0.59 ^a ± 0.03	3.63 ^a ± 0.05
F	*	*	*	*
CV (%)	5.85	1.93	6.21	5.66

Note: Numbers followed by different letters in the same column are different at 5% significance based on the Duncan post hoc test. (*) different at 5% significance.

Table 7. Antagonistic efficiency of *Trichoderma* spp. against the *Lasiodiplodia egyptiaca* L-SA06 strain causing stem-end rot disease in star apples.

Isolate	Antagonistic Efficiency (%)		Antagonistic Diameter Against the <i>Lasiodiplodia egyptiaca</i> L-SA06 Strain (cm)	
	48 h	72 h	48 h	72 h
T-SA03	16.2 ^e	44.7 ^e	0.44 ^e ± 0.01	2.50 ^e ± 0.07
T-SA05	21.1 ^c	59.3 ^a	0.57 ^c ± 0.06	3.27 ^{ab} ± 0.06
T-SA08	19.9 ^{cd}	46.3 ^d	0.54 ^{cd} ± 0.06	2.59 ^d ± 0.03
T-SA14	7.29 ^f	57.8 ^b	0.20 ^f ± 0.01	3.23 ^b ± 0.03
T-SA18	7.79 ^f	46.3 ^d	0.21 ^f ± 0.00	2.59 ^d ± 0.03

Table 7. Cont.

Isolate	Antagonistic Efficiency (%)		Antagonistic Diameter Against the <i>Lasiodiplodia egytiacae</i> L-SA06 Strain (cm)	
	48 h	72 h	48 h	72 h
T-SA19	16.9 ^{de}	58.2 ^{ab}	0.46 ^{de} ± 0.06	3.25 ^{ab} ± 0.03
T-SA25	16.4 ^{de}	52.0 ^c	0.44 ^{de} ± 0.06	2.91 ^c ± 0.04
T-SA26	16.7 ^{de}	45.0 ^e	0.45 ^{de} ± 0.04	2.51 ^e ± 0.11
T-SA30	29.5 ^a	58.6 ^{ab}	0.80 ^a ± 0.08	3.25 ^{ab} ± 0.01
T-SA32	24.6 ^b	59.3 ^a	0.66 ^d ± 0.02	3.31 ^a ± 0.06
F	*	*	*	*
CV (%)	19.7	1.29	11.5	1.07

Note: Numbers followed by different letters in the same column are different at 5% significance based on the Duncan post hoc test. (*) different at 5% significance.

Table 8. Antagonistic efficiency of *Trichoderma* spp. against the *Lasiodiplodia theobromae* L-SA07 strain causing stem-end rot disease in star apples.

Isolate	Antagonistic Efficiency (%)		Antagonistic Diameter Against the <i>Lasiodiplodia theobromae</i> L-SA07 Strain (cm)	
	48 h	72 h	48 h	72 h
T-SA03	11.9 ^e	17.8 ^f	0.35 ^e ± 0.04	0.96 ^f ± 0.09
T-SA05	24.9 ^b	53.2 ^a	0.72 ^b ± 0.03	2.87 ^a ± 0.04
T-SA08	12.6 ^{de}	35.4 ^d	0.37 ^{de} ± 0.04	1.91 ^d ± 0.05
T-SA14	13.6 ^{cde}	35.6 ^d	0.39 ^{cde} ± 0.08	1.92 ^d ± 0.00
T-SA18	13.6 ^{cde}	35.4 ^d	0.39 ^{cde} ± 0.02	1.91 ^d ± 0.01
T-SA19	9.67 ^f	40.0 ^c	0.28 ^f ± 0.02	2.16 ^c ± 0.00
T-SA25	15.7 ^c	36.1 ^d	0.45 ^c ± 0.02	1.95 ^d ± 0.01
T-SA26	14.5 ^{cd}	28.4 ^e	0.42 ^{cd} ± 0.02	1.53 ^e ± 0.27
T-SA30	25.2 ^b	54.1 ^a	0.70 ^b ± 0.01	2.92 ^a ± 0.00
T-SA32	28.8 ^a	49.9 ^b	0.83 ^a ± 0.02	2.69 ^b ± 0.05
F	*	*	*	*
CV (%)	7.02	4.54	6.44	4.55

Note: Numbers followed by different letters in the same column are different at 5% significance based on the Duncan post hoc test. (*) different at 5% significance.

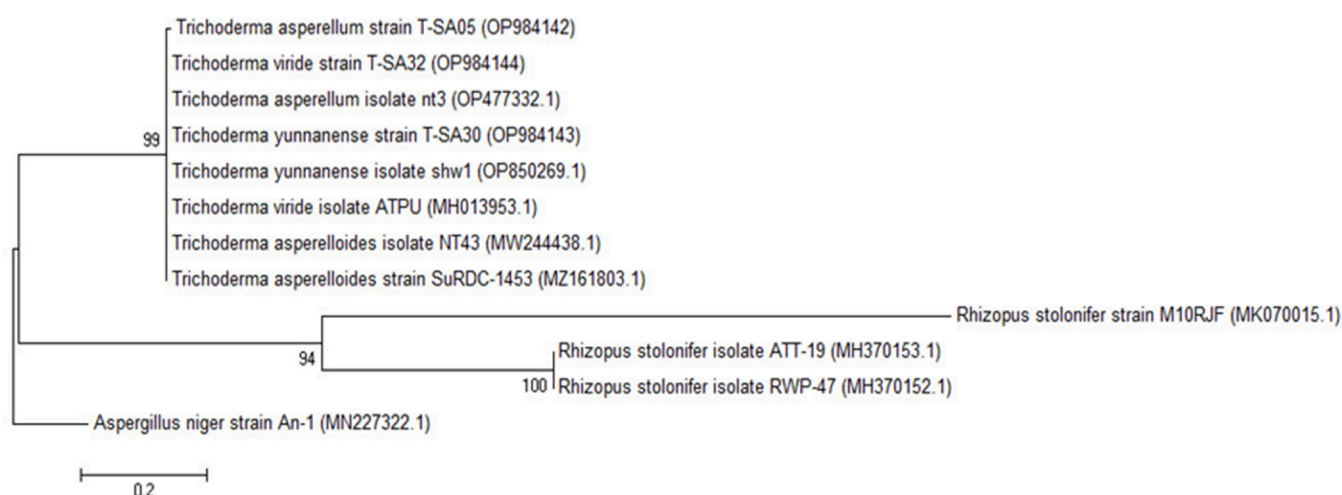


Figure 6. Neighbor-joining phylogenetic trees based on the internal transcribed spacer region sequences of three selected fungi strains compared to the closely related strains in the GenBank database.

The bootstrap analysis percentage levels of 1000 replicates are indicated at each node. The bar represents 0.2 substitutions per nucleotide position. The *Aspergillus niger* An-1 strain was used as the outgroup strain. Access numbers of GenBank sequences are implied in brackets.

4. Discussion

The isolations resulted in 10 strains of pathogenic fungi causing SER in star apples, with a diverse morphology of mycelia (Figure 1). The growth rates of the fungal pathogens were 0.85–1.09 mm h^{−1} at 96 h after incubation (Table 1). This result was in accordance with the growth rates of 12 *Lasiodiplodia* spp. strains isolated from peanut soil, from 13.8 to 14.4 mm day^{−1} [42]. *L. theobromae* fungus was determined as a cause of SER in the Lo Ren star apple [7]. *L. egyptiacae* is a cause of dieback disease in mango [43,44], and *L. venezuelensis* is a pathogen in stems of the Caribbean pine [34]. In the current study, *L. egyptiacae* and *L. venezuelensis* fungi were found to cause SER in star apples (Figure 3). Therefore, this is one of the first studies to detect the *L. egyptiacae* and *L. venezuelensis* strains to be able to cause SER disease in star apples. However, only three fruits were used per treatment, and disease severity, lesion expansion, and variation among fruits were not quantitatively assessed. Therefore, the pathogenicity results should be interpreted as preliminary confirmation of symptom reproduction rather than a comprehensive comparison of virulence among the isolates. Moreover, species of *Lasiodiplodia* can have overlapping colony and conidial characteristics [45], and ITS sequences may provide insufficient variation for resolving closely related taxa [46,47]. Thus, the present assignments require confirmation using detailed conidial measurements and additional loci, such as *tef1-α* and *tub2* [47].

The mycelia of *Trichoderma* spp. grew and covered the backs of the dishes after 4 days of inoculation (Figure 4); they turned light green or dark gray, with rates of 75.6–90.0 mm after 96 h of incubation (Table 2). This was in accordance with Sundaramoorthy and Balabaskar [48], who found that hyphae of *Trichoderma* spp. also had sky fiber forms, lean growth and green color under in vitro conditions. According to Kim et al. [49], the average growth rate of *T. harzianum* was 22.86 mm day^{−1} on PDA media.

The fungi's ability to produce cellulase was determined via the formation of clear zones. Among the isolates selected for antagonistic testing, T-SA05 showed high endoglucanase and exoglucanase activities, whereas T-SA32 showed the highest chitinase activity. The strains of *T. asperellum* T-SA05, *T. yunnanense* T-SA30, and *T. viride* T-SA32 produced chitinase from 0.027 to 0.047 UI mL^{−1} (Table 3). These can be considered mechanisms that antagonize *Lasiodiplodia* spp. causing SER disease in star apples. These characteristics may have contributed to their antagonistic performance because chitin and β-glucans are major structural components of many fungal cell walls, and chitinases and β-1,3-glucanases can participate in mycoparasitic interactions [50,51]. *Trichoderma* spp. secrete extracellular chitinase, which can destroy the cell walls of other fungi [24,52], i.e., chitinase cuts oligomer chitin into chitobiose chitin and glucosamine chitin to fight against pathogenic fungi [53,54]. Particularly, *T. longibrachiatum* GJ-Sp1 and TOP-Co8 fungi antagonize *A. flavus* and *A. niger* fungi based on the activity of chitotriosidase, β-N-acetylglucosaminidase (exochitinase) and chitotriosidase (endochitinase) [53]. The hyphae of *Trichoderma* spp. bind to pathogens and engulf them before hydrolyzing pathogen structures and functions until the pathogens are eliminated. In addition, as reported by Saravanakumar et al. [55], high amounts of cellulase activity enhance cellulose degradation by *Trichoderma* spp. fungi. According to Edison et al. [56], *Trichoderma* produces two types of enzymes, namely, endo-β-glucanase and exo-β-glucanase, that break down glucose segments from the unreduced end of polymers or oligomers, release glucose and degrade cell walls. Endo β-1,3-glucanase randomly breaks the β-1,3-glycosidic bonds along β-glucan chains and creates glucan oligosaccharides as a product [57]. *Trichoderma* spp. decompose cell walls of pathogenic fungi via the combined

activity of chitinase and β -1,3-glucanase, which increases regressive efficiency through cell wall degradation and deformation of pathogenic hyphae and fungal cells [52,58]. However, T-SA30 showed broad antagonistic activity despite not having the highest activity in the enzyme assays. This lack of correspondence indicates that its antagonism was probably not determined solely by the measured extracellular enzymes. Rapid colonization of the culture medium, competition for nutrients and space, secretion of antifungal secondary metabolites, production of volatile compounds, and direct mycoparasitism may also contribute to pathogen suppression by *Trichoderma* [22,24,25,59].

In the dual-culture assays, T-SA05, T-SA30, and T-SA32 showed broad inhibition of the five SER-associated isolates. The greatest inhibition was observed against L-SA03, with antagonistic efficiencies of 80.8–82.4%, whereas lower efficiencies were generally recorded against L-SA07. This isolate-dependent response may reflect differences in pathogen growth rate, nutrient acquisition, secretion of defensive metabolites, sensitivity to *Trichoderma*-derived compounds, or the timing of physical contact between the antagonist and pathogen. Previous studies have similarly shown that antagonistic efficiency varies among *Trichoderma* isolates and target pathogens [60–68]. As reported by Bui et al. [60], *T. asperellum* antagonizes *L. theobromae*, with an AE up to 95.24%. *T. asperellum* also reduces 74.10% of calyx rot in strawberries caused by *L. theobromae* [61]. In addition, according to Bhadra et al. [62], *T. viride* controls *L. theobromae* better than commercial fungicides. In the current study, *Trichoderma* spp. were able to antagonize *Lasiodiplodia* spp. (Figure 5). In detail, *T. yunnanense* T-SA30 antagonized *L. theobromae* L-SA01 at 58.9% (Table 4), *L. venezuelensis* L-SA02 at 66.8% (Table 5), *L. theobromae* L-SA03 at 81.3% (Table 6), *L. egyptiacae* L-SA06 at 58.6% (Table 7) and *L. theobromae* L-SA07 at 54.1% (Table 8). Therefore, this is one of the first studies to determine that *T. yunnanense* can control *Lasiodiplodia* spp. causing SER disease in star apples (Figure 6).

Nevertheless, the current study has several limitations that should be considered. Firstly, the detached-fruit pathogenicity assay included only three fruits per treatment, limiting the precision and generalizability of the pathogenicity observations. Secondly, the antagonistic activity of *Trichoderma* was evaluated only in vitro. In other words, the selected isolates were not applied to intact or detached fruits to determine their capacity to prevent or reduce SER. Thirdly, taxonomic placement was based on ITS sequences alone. Fourthly, no formal correlation analysis was conducted between enzyme activities and antagonistic efficiencies. Finally, other possible mechanisms, including antifungal metabolites, volatile compounds, nutrient competition, and direct mycoparasitism, were not measured. Therefore, future studies should use multilocus identification, larger pathogenicity experiments, microscopy of antagonist–pathogen interactions, metabolite and enzyme analyses under dual-culture conditions, and postharvest fruit trials before practical biological control can be concluded.

5. Conclusions

The pathogenic selection resulted in five indigenous fungal strains that led to SER disease in star apples, including *Lasiodiplodia* sp. L-SA01, L-SA03 and L-SA07, *L. venezuelensis* L-SA02, and *L. egyptiacae* L-SA06. The antagonistic selection resulted in three indigenous strains, including *Trichoderma asperellum* T-SA05, *T. yunnanense* T-SA30 and *T. viride* T-SA32, that controlled the five strains *L. theobromae* L-SA01, *L. venezuelensis* L-SA02, *L. theobromae* L-SA03, *L. egyptiacae* L-SA06 and *L. theobromae* L-SA07. The *T. asperellum* T-SA05, *T. yunnanense* T-SA30 and *T. viride* T-SA32 strains had AEs against the L-SA01, L-SA02, L-SA03, L-SA06 and L-SA07 pathogenic strains of 55.4–58.9, 64.9–69.6, 80.8–82.4, 58.6–59.3 and 49.9–54.1%, respectively. The selected strains of *Trichoderma* spp. were able to produce cell wall-hydrolyzing enzymes, including chitinase, exo- β -1,3-glucanase and endo- β -1,3-

glucanase, which were considered as mechanisms that control pathogenic fungi under in vitro conditions. However, although the selected isolates showed different extracellular enzyme profiles, these profiles did not fully explain their antagonistic performance, suggesting the involvement of additional mechanisms. Moreover, they have not yet demonstrated control of SER under commercial or in vivo application conditions. Last but not least, multilocus analyses and detailed morphological characterization are required for definitive species-level identification.

Author Contributions: Conceptualization, N.Q.K. and D.T.X.; methodology, L.T.Q. and D.T.X.; validation, D.T.X. and L.T.Q.; formal analysis, N.Q.K., T.K.H., L.T.M.T., N.T.P., D.T.X. and L.T.Q.; investigation, N.Q.K., T.K.H., L.T.M.T., N.T.P., D.T.X. and L.T.Q.; data curation, N.Q.K., T.K.H. and L.T.Q.; writing—original draft preparation, N.Q.K.; writing—review and editing, L.T.Q. and D.T.X.; visualization, T.K.H., L.T.M.T. and N.T.P.; supervision, D.T.X. and L.T.Q.; project administration, L.T.Q. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The original contributions presented in this study are included in this article. Further inquiries can be directed to the corresponding author.

Acknowledgments: The authors sincerely appreciate the Edible and Medicinal Mycology Laboratory (Faculty of Crop Science, College of Agriculture, Can Tho University), founded by sponsors from ODA, which provided equipment, chemicals and materials for this study to be completed.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AE	Antagonistic efficiency
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
CFU	Colony-forming unit
CMC	Carboxymethyl cellulose
CV	Coefficient of variation
DAI	Days after inoculation
DNA	Deoxyribonucleic acid
ITS	Internal transcribed spacer
L-SA	<i>Lasiodiplodia</i> isolate from star apple
MEGA	Molecular Evolutionary Genetics Analysis
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
SER	Stem-end rot
T-SA	<i>Trichoderma</i> isolate from star apple rhizosphere soil
TE	Tris-EDTA buffer
TSM	<i>Trichoderma</i> selective medium
UI	Unit of enzyme activity

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