

The effectiveness of *Trichoderma asperellum* strain DN7.5 in controlling bacterial wilt disease caused by *Ralstonia pseudosolanacearum* T2C-Rasto on cucumbers *in vivo* conditions

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ABSTRACT

Bacterial wilt disease, caused by *Ralstonia solanacearum*, causes significant loss to cucumber production in the Mekong Delta. In this study, the antibacterial activity of 95 strains against *Ralstonia pseudosolanacearum* strain T2C-Rasto was tested by the agar well method. Under laboratory conditions, three *Trichoderma* strains involving DN7.5, DN2.6, and DN30.7 showed strong inhibitory activity against *Ralstonia pseudosolanacearum* strain T2C-Rasto with effectiveness of 74.29, 67.63, 67.63%, respectively. Under *in vivo* conditions, *Trichoderma* strain DN7.5 showed the highest effectiveness against *Ralstonia pseudosolanacearum* strain T2C-Rasto, reducing disease incidence by 26.67% after 14 days without affecting cucumber plant growth. The ITS (internal transcribed space) + 26S rRNA gene region of *Trichoderma* strain DN7.5 showed 98.13 - 98.38% sequence similarity with ITS sequence of *Trichoderma asperellum* strains (MF363050.1 - OM515036.1). Generally, *Trichoderma* strain DN7.5 has the potential for further research on the mechanisms of suppression against *Ralstonia pseudosolanacearum* strain T2C-Rasto.

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1. Introduction

Cucumbers in the Mekong Delta are susceptible to bacterial wilt disease caused by *R. solanacearum* (Timothy, 2000; Emmanuel et al., 2002; Le et al., 2023), especially after the second crop (Timothy, 2000; Le et al., 2021). *R. solanacearum* infects plant roots, vascular bundles, and causes plant death. To prevent and treat this disease, a substantial number of soil treatment chemicals are required, which are costly, ineffective, and dangerous (Marc et al., 2015; Roop & Jagtap, 2017; Le et al., 2021). Using beneficial microbes to resist and compete with harmful bacteria is highly valued. (Marc et al., 2015; Shashitu, 2021; Sutarman, 2021). *R. pseudosolanacearum* strains T2C-Rasto, isolated from wilt *Cucumis sativus*, in An Giang province, Mekong Delta - Southwest Vietnam. T2C-Rasto can 100% re-infect cucumber plants *in vivo* after 10 days of infection. Strain T2C-Rasto was used to test the antagonistic effects of fungal strains both *in vitro* and *in vivo*. T2C-Rasto was uploaded draft gene sequencing (accession number JAHWRH010000000.1) (Le et al., 2021; 2023).

Trichoderma controls diseases by competing for nutrients, producing antibiotics, and activating plant immunity (Peng et al., 2015; Yan et al., 2021; Zhou et al., 2021). In studies, the combination of *T. viride* + *Streptomyces* sp. + *Pseudomonas fluorescens* reduced wilt disease incidence by 44.85 - 50.09% in potatoes (Rosyidah et al., 2013). *Trichoderma* T1 reduced disease incidence by over 61.66% in tomatoes in the field (Kariuki et al., 2020). *T. harzianum* reduced the incidence of wilt disease on brinjal plants by 33.33% in field conditions (Qulsum et al., 2023), and 40% on eucalyptus plants in pots (Gomes et al., 2023). The combined treatment of 6% leaf waste biochar + *T. harzianum* significantly reduced the disease index by 90% (Ahmad et al., 2024).

T. asperellum effectively reduces wilt disease on tomato plants by 50% in greenhouses, and 46.83 - 52.75% in fields (Narasimha et al., 2013; 2018), and on ginger plants by 13.33% in greenhouses (Islam et al., 2020). *T. asperellum* T34 reduced wilt disease by 10.7 - 26.5% on potato plants in greenhouses and 26.6 - 36.6% in fields (Bereika et al., 2020). *T. asperellum* T34 reduced wilt disease by 10.7 - 26.5% on potato plants in greenhouses and 26.6 - 36.6% in fields (Bereika et al., 2020). *T. asperellum* enhances canola root growth through auxin production (Ada et al., 2010) and converts minerals into absorbable nutrients (Xinde et al., 2011). However, its efficacy against bacterial wilt in cucumbers remains unexplored.

In this study, we isolated and selected fungal strains capable of resisting strain T2C-Rasto both *in vitro* and *in vivo*. Strain DN7.5 demonstrated that it efficiently controlled the disease in cucumbers.

2. Materials and Methods

2.1. Materials

Strain T2C-Rasto causes bacterial wilt disease on cucumbers in the Mekong Delta (taken from the laboratory, National Center for Technological Progress, Ho Chi Minh Branch) (Le et al., 2023). *Trichoderma* strains were isolated from samples of primary forest land, healthy cucumber garden land, and long-term uncultivated land in the Mekong Delta.

The culture medium for *Trichoderma* was potato dextrose agar (PDA) (containing 200 g/L potatoes; 20 g/L Glucose and 20 g/L agar).

The culture medium for strain T2C-Rasto and the antibacterial testing medium was Sucrose Peptone Agar (SPA) (containing 5 g peptone; 20 g sucrose; 0.5 g K₂HPO₄; 0.25 g MgSO₄·7H₂O; 18 g agar and pH 7.0).

2.2. Methods

Isolation of *Trichoderma*

These soil samples were diluted and slided on PDA medium, then were placed in an incubator at 28°C. The colonies visible on the medium's surface were monitored, and individual colonies were separated on PDA medium after cultured 3 - 10 days. The *Trichoderma* was identified by their morphology (Bui & Nguyen, 2000).

Evaluating the efficacy of antagonists against T2C-Rasto using agar well and agar well diffusion methods

Agar well method: *Trichoderma* was cultured on a PGA medium at 28°C for 4 days and punched into 7 mm diameter colonies. T2C-Rasto was cultured in sucrose peptone broth (SPB) medium at 28°C for 24 h, diluted to $\times 10^8$ CFU/mL ($OD_{600} = 0.1$), and plated 0.1 mL on SPA medium. Wells of 7 mm diameter were created in the SPA medium, and agar bars with *Trichoderma* were placed in the wells. The plates were then incubated at 28°C for 4 days to observe the growth rings of T2C-Rasto. The experiment was replicated three times for each *Trichoderma* strain. Effective inhibition = $100 \times (C - T/C)$, (C = Inhibited T2C-Rasto colony diameter with sterile ddH₂O treatment) and (T = Inhibited T2C-Rasto colony diameter with *Trichoderma* treatment) (Le et al., 2021). Three *Trichoderma* strains (DN7.5, DN2.6, and DN30.7) were selected to test their antagonistic effects against T2C-Rasto using the agar well diffusion method. These strains were also evaluated for their ability to control wilt disease *in vivo* (Narasimha et al., 2013; Le et al., 2021). *Trichoderma* strains were categorized based on their antagonistic effectiveness levels: < 20% (no resistance), > 20 - 40% (weak resistance), > 40 - 60% (average resistance), > 60% (high resistance) (Tran & Pham, 2012).

Agar well diffusion method: The *Trichoderma* strains were cultured in potato dextrose broth (PGB) medium for 10 days at 28°C. The liquid was centrifuged at 12,000 g for 10 min at 28°C, and the cell-free supernatants were collected. T2C-Rasto was cultured in SPB medium, diluted to ($OD_{600} = 0.1$), and plated 0.1 mL on SPA medium. Wells were created in the agar, and 0.1 mL of the *Trichoderma* liquid (without cells) was added to each well. The experiment was replicated three times for each strain, and the diameter of the inhibition zone was calculated by subtracting the well diameter (7 mm) from the antagonistic ring diameter (Narasimha et al., 2018).

Testing the efficacy of biocontrol agents *in vivo*

Inoculum preparation: T2C-Rasto was cultured in SPB medium for 24 h at 28°C (*Trichoderma* was cultured in PDB medium for 7 days at 28°C). The biomass was collected by centrifugation at 1,2000 g for 10 min at 28°C and diluted in distilled water to $OD_{600} = 0.1$ ($\times 10^8$ CFU/ml T2C-Rasto), and $OD_{600} = 0.5$ ($\times 10^8$ CFU/mL *Trichoderma*).

Soil preparation: Soil samples from the Mekong Delta were dried, ground to a 6 mm pore size, and mixed with husk at a 10:1 ratio. The soil mix was sterilized in Nylon plastic bags using autoclaving at 121°C for 1 h. This sterilized soil was used for sowing cucumber seeds in 50 mm diameter plastic cups and planting cucumber plants in pots measuring 40 x 100 x 20 cm.

Three-day-old cucumber plants preparation: Cucumber seeds of the Ngoc Long Chau variety are commonly grown in the Mekong Delta due to their high commercial value but low resistance to the wilt disease. The study involved planting cucumber seeds in individual plastic cups with 10 g of soil. Three-day-old cucumber plants with one young leaf were used for testing the resistance to wilt disease.

Evaluation of *Trichoderma* against T2C-Rasto: The experiments were conducted three times, with one concentration in each set of five pots. Three-day-old cucumber plants were placed in plastic pots with 3 kg of soil. After 5 days, antagonistic *Trichoderma* was applied at concentrations of 10^7 , 10^5 , and 10^3 CFU/mL (10 mL of each *Trichoderma* concentration was added to each pot), along with positive (T2C-Rasto) and negative controls (sterile distilled water). After 7 days, 10 mL of strain T2C-Rasto (10^8 CFU/mL) was added to each pot. Disease incidence (DI) was calculated as the percentage of infected plants at 7, 14, and 21 days: $DI (\%) = (\text{number of infected plants} \times 100 / \text{total number of tested plants})$ (Narasimha et al., 2013; 2018).

***Trichoderma* identification method using the ITS (internal transcribed space) + 26S rRNA gene (D1/D2 region) sequencing:** The biomass strain DN75 was sequenced at Macrogen in Korea after being grown in 25 mL of PDB medium at 25°C for 4 days. Genomic DNA was extracted using the cetyltrimethylammonium bromide procedure and amplified with ITS 1

(5'-TCCGTAGGTGAACCTGCGG-3') and LR3 (5'-CCGTGTTTCAAGACG GG-3') primers. PCR cycling conditions included pre-denaturation at 95°C for 5 min, denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and primer extension at 72°C for 1 min for 40 cycles using the Biorad T100 thermal cycler. The PCR product was sequenced using ABI 3730XL sequencers. Nucleotide sequences were compared to GenBank using NCBI's BLAST, and phylogenetic analyses were performed in MEGA X (Macrogen Inc., Macrogen Europe, Amsterdam, Netherland).

3. Results and Discussion

Isolation of *Trichoderma*

A total of 95 fungi were isolated from 50 soil samples collected in the Mekong Delta region. Strains DT1 to DT68, displayed colony features to *Trichoderma*, with colors ranging from white to dark green, yellow-green, or white-green when spores were produced (Figure 1).

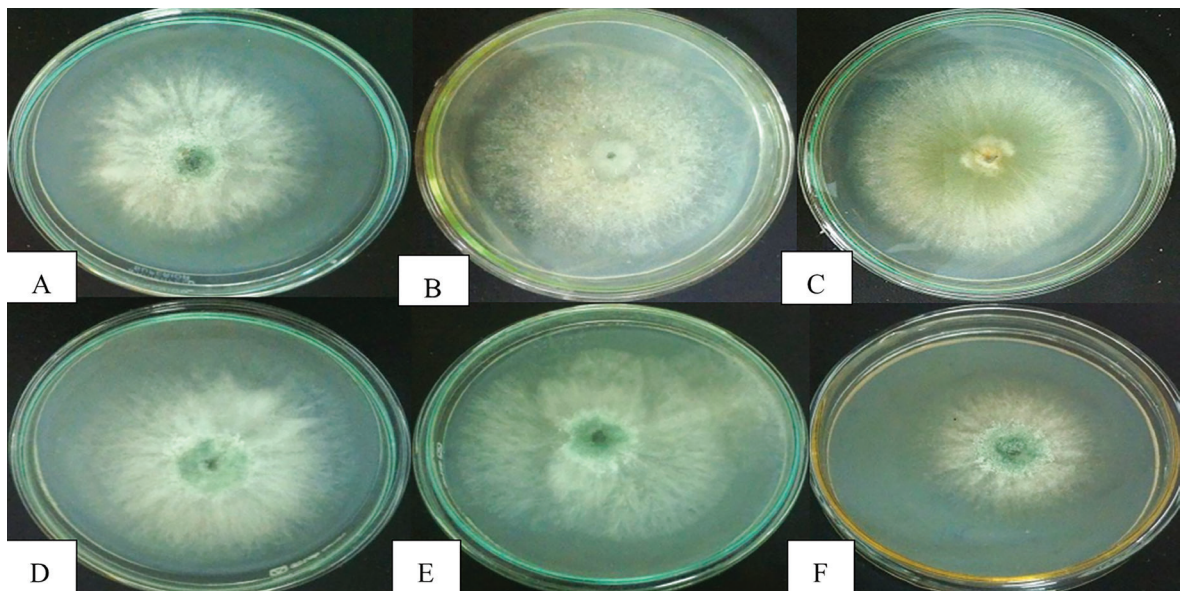


Figure 1. *Trichoderma* strains were isolated on PDA medium after 3 days of culture at 28°C. A) DN2.6; B) DN7.5; C) DN30.7; D) DN1.3; E) DN4.2; F) DN35.4. PDA: Potato dextrose agar.

The analysis of isolation showed different quantities of *Trichoderma* strains in soil samples from various locations, ranging from 1.1 to 2.5 x 10⁶ CFU/g (Table 1). Dong Thap soil samples had lower fungal microbial density (CFU/g)

compared to other locations, suggesting factors such as soil type, nutrient levels, weather conditions, and agricultural practices influence these variations.

Table 1. Soil samples from the Mekong Delta were used to isolate *Trichoderma* strains

Order	Sampling location	Number of samples	Soil type	Trichoderma density (CFU/g)	Number of strains
1	An Giang	40	Primary forest land, healthy cucumber garden land, long-term uncultivated land	1.5 * 10 ⁶	78
2	Dong Thap	5	healthy cucumber garden land, long-term uncultivated land	1.1 * 10 ⁶	9
3	Tien Giang	3	healthy cucumber garden land, long-term uncultivated land	2.5 * 10 ⁶	3
4	Long An	2	healthy cucumber garden land, long-term uncultivated land	1.2 * 10 ⁶	5
Total		50			95

Note: CFU: Clony forming unit.

Mycelia and spores of 95 *Trichoderma* were observed under a microscope at 40X magnification (Figure 2). The spores displayed different shapes such as spherical, elliptical, oval, or rectangular, with most being smooth. Conidiophore-producing spores were elongated with callous-shaped bodies, either in clusters or

individually. The branches grew perpendicular or nearly perpendicular to the main axis, often branching symmetrically. Spores were typically long and narrow with a slim neck, while the vase form was broader in the center and tapered to a thin base at the top.

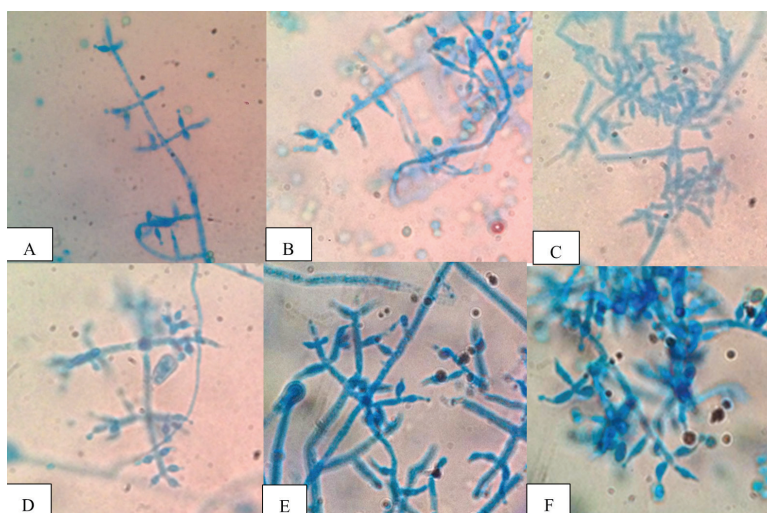


Figure 2. Morphology of different *Trichoderma* strains on PDA medium after 4 days at 28°C under the microscope at 40X magnification. A) Strains DN2.6; B) DN7.5; C) DN30.7; D) DN1.3; E) DN4.2; F) DN35.4. PDA: Potato dextrose agar.

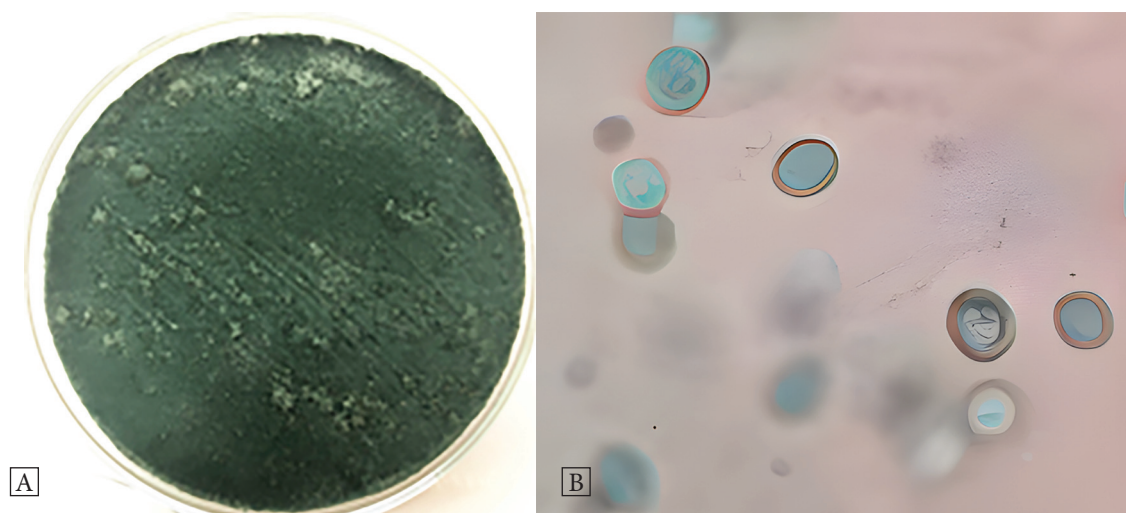


Figure 3. The color (a) and morphology (b) of spores from *Trichoderma* strain DN7.5 (size spores length x breadth: 1.85 - 2.90 x 1.05 - 2.25 μm).

Store fungal strains in subculture with 20% glycerol at -80°C . Preserve strains through freeze-drying to test their efficacy against strain T2C-Rasto *in vivo* conditions.

Evaluating the efficacy of antagonists against *R. pseudosolanacearum* T2C-Rasto

Table 2 displayed the antagonistic levels of *Trichoderma* strains: 12 strains (12.63%) were highly antagonistic, 27 strains (28.42%) showed average antagonism, 18 strains (18.95%) had weak antagonism, and 38 strains (40.00%) were non-antagonistic.

Table 2. Antagonistic activity of *Trichoderma* sp. against *R. pseudosolanacearum* T2C-Rasto

Resistance level	Inhibitory effect (%)	Number of strains	Ratio (%)
No resistance	< 20	38	40.00
Weak resistance	> 20 - 40	18	18.95
Average resistance	> 40 - 60	27	28.42
High resistance	> 60	12	12.63
Total		95	100

Table 3 presented the inhibitory impact of 12 *Trichoderma* antagonistic fungal strains on T2C-Rasto, with inhibitory efficacy between 60.94 - 74.29%. No notable variance was observed between the strains at the $P < 0.05$ significance level. Three *Trichoderma* strains DN7.5, DN2.6, and DN30.7 expressed the ability to inhibit the

growth of T2C-Rasto with inhibitory efficacy of 74.29, 67.63, and 67.63%, respectively. Among the strains, *Trichoderma* strain DN7.5 demonstrated the highest inhibitory effect at 74.29% after 5 days. *Trichoderma* strain DN7.5 grows rapidly, fully covering a Petri dish within 5 days (Figure 3).

Table 3. Antagonistic activity of *Trichoderma* against *R. pseudosolanacearum* T2C-Rasto using agar well method

Order	Antagonistic <i>Trichoderma</i> strains	Inhibited T2C-Rasto colony diameter (mm)	Inhibitory effect (%)
1	DN1.3	13.67 ^{ab}	60.94 ^{ab}
2	DN2.6	11.33 ^{ab}	67.63 ^{ab}
3	DN4.2	13.00 ^{ab}	62.86 ^{ab}
4	DN7.5	9.00 ^a	74.29 ^a
5	DN8.1	11.67 ^{ab}	66.66 ^{ab}
6	DN8.9	12.67 ^b	63.80 ^b
7	DN10.3	12.33 ^{ab}	64.77 ^{ab}
8	DN22.5	11.67 ^{ab}	66.66 ^{ab}
9	DN30.1	13.67 ^{ab}	60.94 ^{ab}
10	DN30.7	11.33 ^{ab}	67.63 ^{ab}
11	DN33.6	11.67 ^{ab}	66.66 ^{ab}
12	DN35.4	13.67 ^{ab}	60.94 ^{ab}
13	Control	35.00 ^c	00 ^c

Note: ^{abc}, Mean values in the same column followed with the same letter are not significantly different ($P < 0.05$, $CV = 0.47$, $F\text{-Value} = 9.15$).

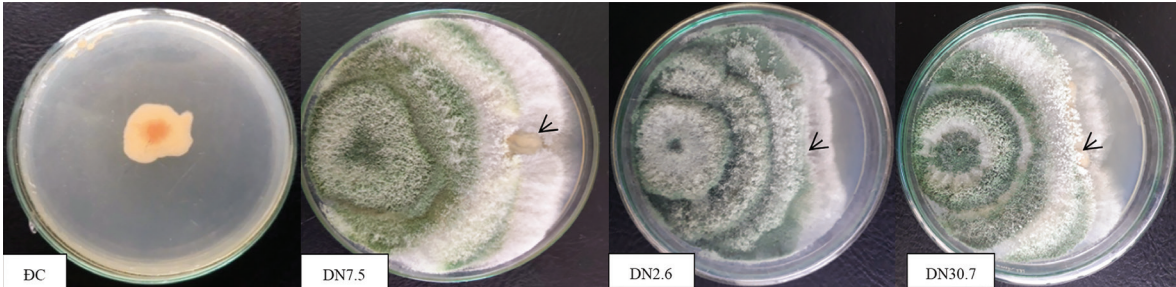


Figure 4. *Trichoderma* strains with the highest antagonistic activity against T2C-Rasto were identified: A) DN7.5, B) DN2.6, C) DN30.7. (T2C-Rasto; Black arrow symbol ↙).

Three *Trichoderma* strains (DN7.5, DN2.6, and DN30.7) exhibited strong antagonistic effects against T2C-Rasto by agar well method as shown in Figure 4. The production of the

antibacterial metabolites by these strains was confirmed using the agar well diffusion method, revealing their ability to produce antibacterial compounds (Figure 5).

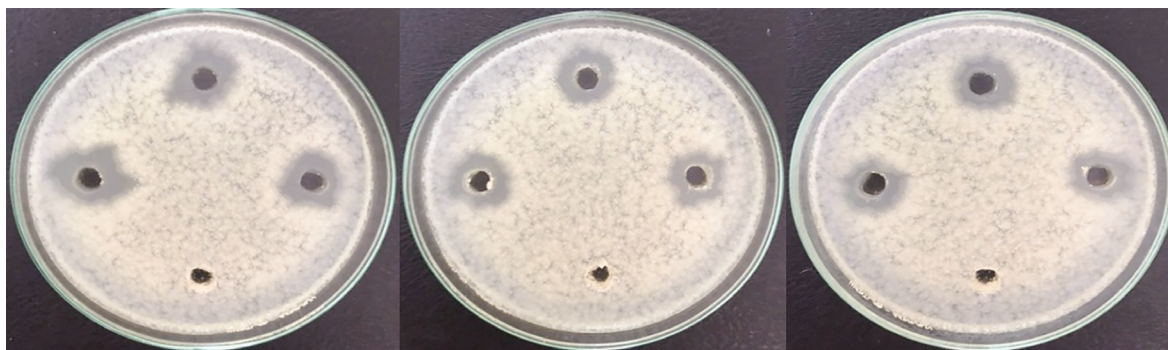


Figure 5. *Trichoderma* strains DN7.5, DN2.6, and DN30.7 exhibited antibacterial against T2C-Rasto using the agar well diffusion method.

Three strains DN2.6, DN7.5, and DN30.7 were chosen for testing their efficacy in controlling bacterial wilt disease on cucumber plants.

Evaluating the effects of biocontrol agents *in vivo*

Table 4 and Figure 6 showed the test results for strains (DN2.6, DN7.5, and DN30.7) in controlling wilt disease. After 7 days of T2C-Rasto infection, the *Trichoderma*-treated plots

showed a 6.66 - 33.00% decrease in disease incidence compared to the positive control plot. *Trichoderma* strain DN7.5 showed the greatest reduction in disease incidence at 33.33% at a density of 10^7 CFU/g.

After 14 days of T2C-Rasto infection, wilt disease incidence decreased by 0.00 - 26.67%. Strain DN7.5 at a density of 10^7 CFU/g showed the highest reduction at 26.67%. The growth of cucumber plants was unaffected by strain DN7.5.



Figure 6. Evaluation of *Trichoderma* strains for controlling bacterial wilt disease *in vivo*. (A) Test model, (B) Test results of strain DN7.5.

Table 4. The effectiveness of *Trichoderma* strains in controlling wilt disease *in vivo*

Experience	Density (CFU/g)	Disease incidence (%)		Growth of cucumber plants
		After 7 days of T2C-Rasto infection	After 14 days of T2C-Rasto infection	
DN 2.6	10 ³	66.67 ^c	100.00 ^d	-
	10 ⁵	60.00 ^{bc}	86.67 ^{bcd}	
	10 ⁷	60.00 ^{bc}	80.00 ^{bc}	
DN 7.5	10 ³	66.67 ^c	93.33 ^{cd}	-
	10 ⁵	53.33 ^{bc}	80.00 ^{bc}	
	10 ⁷	40.00 ^b	73.33 ^b	
DN 30.7	10 ³	66.67 ^c	100.00 ^d	-
	10 ⁵	66.67 ^c	86.67 ^{bcd}	
	10 ⁷	53.33 ^{bc}	80.00 ^{bc}	
Positive control		73.33 ^c	100.00 ^d	
Negative control		0.00 ^a	0.00 ^a	
		CV = 0.41, F value = 5.73	CV = 0.36, F value = 7.75	

Note: ^{abcd}, Mean values in the same column followed with the same letter are not significantly different ($P < 0.05$). (+): Impacting plant growth; (-): No impacting plant growth.

The experiment revealed that strain DN7.5, at a density of 10⁷ CFU/g, effectively controlled bacterial wilt disease without impacting cucumber plant growth, with a Disease incidence of 73.33% and biological control efficiency of 26.67%. Shashitu. (2021) discovered that *T. asperellum* strain AAURB20 efficiently controls bacterial wilt disease on tomatoes, with a disease incidence of 13.33% and a biological control efficiency of 72.22%. Narasimha et al., (2018) discovered that *T. asperellum* T4 and T8 decreased the disease incidence on tomato plants by 50% in greenhouses. Furthermore, it reduces disease incidence circumstances, decreasing from 10.70 - 26.50% on potato plants in the greenhouse (Bereika et al., 2020).

Identification of *Trichoderma* strains DN7.5 using sequence ITS + 26 rRNA gene (D1/D2 region) region

The 1122-base PCR product of the ITS + 26 rRNA region follows as:

The sequence was compared to the NCBI (National Center for Biotechnology Information) database by nucleotide Blast, which revealed that the strain was *Trichoderma asperellum* DN7.5 (98.13 - 98.38% similarity with ITS sequence of *Trichoderma asperellum* strains: CEN768, CEN698, CEN201, FT101, and strain DQ-1, with Query Length of 1122 nucleotides).

The phylogenetic tree was constructed using MEGA X software and the ITS + 26S rRNA gene sequences from the NCBI Gene database. Analytical results showed the strain DN7.5, discovered in the Mekong Delta, belongs to the species *T. asperellum* at a close evolutionary relationship with a distance difference of < 0.001 (Figure 7).

T. asperellum is a beneficial strain that interacts with plant roots, soil, and leaves, enhancing growth potential, disease resistance and tolerance to abiotic stresses (Narasimha et al. 2018; Bereika et al., 2020; Islam et al., 2020). The study highlights the safety and effectiveness

of *T. asperellum* strain DN7.5 in controlling bacterial wilt disease in cucumber plants in the Mekong Delta. This strain shows potential for further research on its antagonistic mechanisms against T2C-Rasto.

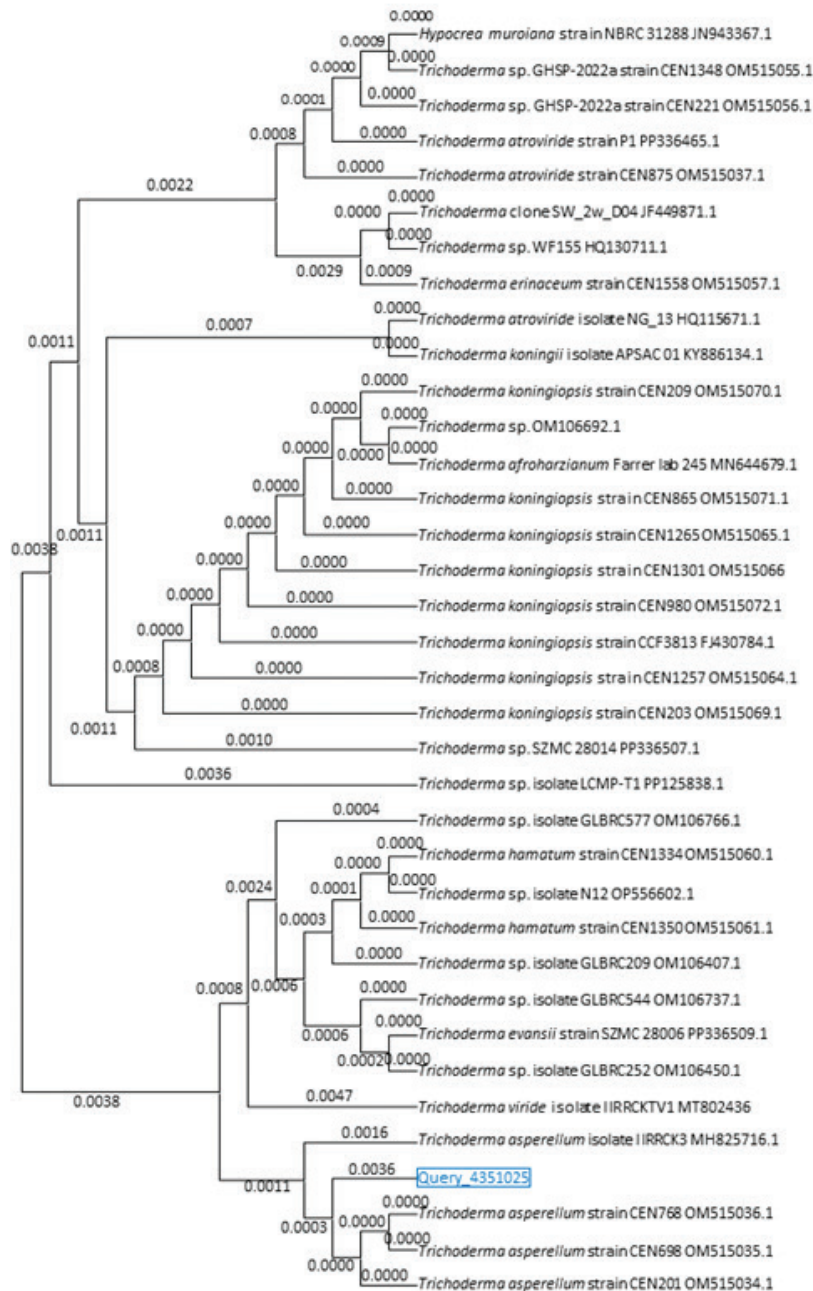


Figure 7. The phylogenetic tree of ITS + 26S rRNA gene sequences from NCBI Gene Bank was constructed using the Neighbor-Joining method in MEGA X. ITS: Internal transcribed space.

4. Conclusions

95 *Trichoderma* strains were isolated from the Mekong Delta, with 12 strains showing strong antagonism to *R. pseudosolanacearum* T2C-Rasto. Three *Trichoderma* strains (DN7.5, DN2.6, and DN30.7) exhibited the highest antibacterial activity against T2C-Rasto, with inhibitory effects of 74.29, 67.63 and 67.63%, respectively, in lab tests. The strain DN7.5 effectively controlled bacterial wilt disease in greenhouse conditions, reducing the disease incidence by 26.67% without affecting cucumber plant growth. Molecular biological identification confirmed the strain as *Trichoderma asperellum* strain DN7.5.

Conflict of interest

This study is original and has not been published elsewhere, and there are no conflicts among the authors.

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