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Enhancement of Damping-Off Disease Control in Tomatoes Using Two Strains of *Trichoderma asperellum* Combined with a Plant Immune Stimulant

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Abstract: Damping-off disease, caused by *Pythium aphanidermatum*, significantly impacts tomato production. This study explored the potential of a two-pronged approach for enhanced biocontrol: combining two antagonistic *Trichoderma asperellum* strains (CB-Pin-01 and NST-009) with a plant immune stimulant ($CaCO_3$). Laboratory assays demonstrated strong individual efficacy of both *Trichoderma* strains against *P. aphanidermatum*, with significant growth inhibition and overgrowth capabilities. Importantly, scanning electron microscopy confirmed their compatibility. Greenhouse experiments revealed that the combined application of *Trichoderma* strains and $CaCO_3$ achieved the most significant reduction in disease incidence (17.78%) compared to the control (66.55%). Furthermore, this treatment resulted in 100% root colonization by *Trichoderma* and the highest population density in the soil (6.17×10^7 CFU g^{-1}), suggesting the immune stimulant's role in promoting beneficial microbe establishment. These findings highlight the potential of this combined strategy as a sustainable and effective approach for managing damping-off disease in tomatoes.

Keywords: damping-off disease; tomato; *Trichoderma asperellum*; biocontrol agent; plant immune stimulant; synergistic effect; sustainable disease control

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1. Introduction

Tomatoes (*Lycopersicon esculentum* Mill) are a vital fruit crop cultivated worldwide and prized for their versatility in culinary applications. They are a rich source of essential vitamins and minerals, including vitamin C, potassium, and folate, and contain lycopene, an antioxidant linked to numerous health benefits [1]. However, a significant threat lurks beneath the soil: damping-off disease [2].

Caused primarily by soilborne pathogens like *Pythium* spp., *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Fusarium* spp., damping-off disease can devastate tomato crops from seed to seedling. It manifests in two stages: pre-emergence damping-off and post-emergence damping-off. In pre-emergence, seeds fail to germinate, leaving empty patches in seedling trays. Post-emergence affects established seedlings, causing soft, discolored areas, typically brown, black, or water-soaked, on the stem near the soil line. Affected seedlings appear weak and stunted and may eventually wilt and collapse. The presence of white, fuzzy fungal growth on the stem base or surrounding soil further confirms the pathogen's presence [2–6].

Traditional control methods like chemical fungicides (azoxystrobin, Metalaxyl-M, and pyraclostrobin) or soil sterilization pose significant drawbacks. Chemical use increases production costs, fosters resistance to pathogens, and harms the soil microbiome, impacting long-term crop health [7,8].

Seeking a sustainable solution, biological control using beneficial organisms like *Trichoderma* spp. emerges as a promising alternative. These fungi thrive in soil and exhibit potent antagonistic properties against plant pathogens, colonizing both plant surfaces and the rhizosphere (the soil zone around roots) to provide multifaceted disease suppression [9–11]. While effective, *Trichoderma* spp. may benefit from optimization strategies. This study explores the potential of combining multiple *Trichoderma asperellum* strains with complementary strengths, augmented by a plant immune stimulant, calcium carbonate ($CaCO_3$), to further enhance disease-fighting capabilities.

Trichoderma species are biocontrol agents utilized to combat various plant diseases, employing direct and indirect mechanisms to target plant pathogens and fortify plant health. In terms of direct mechanisms, *Trichoderma* spp. exhibit robust competitive prowess, vigorously vying for essential resources such as carbon, nitrogen, and space, thereby outcompeting plant pathogens and thwarting their growth within the rhizosphere [12,13]. Moreover, through mycoparasitism, *Trichoderma* launches a multi-step assault on pathogens, commencing with the identification and subsequent attachment to the pathogen's cell wall, followed by enzymatic degradation facilitated by cell wall degrading enzymes (CWDEs) like glucanases and chitinases, ultimately culminating in nutrient absorption from the compromised pathogen [14,15]. Concurrently, through antibiosis, *Trichoderma* deploys an arsenal of antimicrobial compounds such as alkyl pyrones, isonitriles, and polyketides, orchestrating a chemical onslaught that curtails the growth and proliferation of pathogenic fungi in their vicinity [16]. Conversely, indirect mechanisms encompass root colonization, wherein *Trichoderma* establishes a protective shield around plant roots, impeding pathogen access and fortifying the plant against infection [17]. Additionally, elicitors released by *Trichoderma* are involved in the induction of the plant's defense mechanisms against pathogen infection through the production of pathogenesis-related (PR) proteins and enzymes [18,19]. In addition, some strains of *Trichoderma* facilitate plant growth by enhancing nutrient uptake, producing growth-promoting hormones such as auxins and cytokinins, and improving stress tolerance [17].

Calcium carbonate ($CaCO_3$), known as lime, might hold promise in boosting tomato seedlings' natural defenses against disease. This theory is backed by research exploring its use in plant disease control. For instance, studies have shown that seed treatment with a combination of calcium carbonate and *Bacillus amyloliquefaciens* PMB05 bacteria effectively combats black rot disease in cabbage [20]. Additionally, another study revealed that biosynthesized calcium carbonate nanoparticles ($CaCO_3$ NPs) possess moderate antifungal properties against common fungal diseases that plague tomatoes [21].

This research focuses on two highly regarded *Trichoderma asperellum* strains, NST-009 (Walailak University, Nakhon Si Thammarat, Thailand) and CB-Pin-01 (Kasetsart University Kamphaeng Saen Campus, Nakhon Pathom, Thailand), known for their effectiveness against various plant diseases [22–26]. Through comprehensive laboratory and greenhouse trials, these strains' capacity was evaluated, in combination with $CaCO_3$, to control damping-off disease in tomatoes caused by *P. aphanidermatum*. Additionally, their performance was compared to that of Metalaxyl, a commonly used chemical fungicide. By investigating this combined approach, the aim will be to develop a more sustainable and effective strategy for managing damping-off disease and safeguarding the health and productivity of tomato production systems.

2. Materials and Methods

2.1. Isolation and Classification of the Tomato Damping-Off Pathogen

Pythium aphanidermatum, the causal agent of damping-off disease, was isolated from symptomatic tomato root tissue. A sterile section of tissue, encompassing the border between diseased and healthy areas, was excised aseptically using a scalpel disinfected with 70% ethanol. To eliminate surface microbes, the tissue underwent surface disinfection by immersion in a 10% sodium hypochlorite (NaOCl) solution for 1 min. Following disinfection, the tissue was rinsed three times with sterile distilled water to remove residual

NaOCl. Potato Dextrose Agar (PDA) culture medium was prepared according to standard protocols and dispensed into Petri dishes. Two tissue pieces were placed in each dish (totaling 10 pieces across 5 Petri dishes). The sealed Petri dishes were incubated at 28 ± 2 °C under dark conditions for optimal *Pythium* growth. After incubation, fungal colonies emerging from the plant tissue were observed and analyzed under both light microscopy (compound microscope) and field emission scanning electron microscopy (FE-SEM). Morphological characteristics of the colonies (e.g., hyphal growth pattern, color, and sporulation) were used for species classification. These characteristics were compared to descriptions of known *Pythium* spp. documented in scientific literature to ensure accurate identification. Isolated fungal cultures were maintained on PDA slants at 4 °C for further experimentation [8,27].

2.2. Pathogenicity Assay of Isolated Pythium aphanidermatum

To assess the disease-causing ability of the isolated *P. aphanidermatum*, a pathogenicity assay was conducted. Fungal cultures were grown on PDA at 28 ± 2 °C under dark conditions until actively growing. Approximately 0.5 cm × 0.5 cm pieces containing actively growing fungal mycelium (e.g., PDA plugs) were used to prepare the inoculum. These fungal plugs were mixed with sterile distilled water at a 1:500 mL ratio (one culture dish per 500 mL of water) to ensure proper dispersion of fungal propagules for inoculation. Germ-free potting soil, specifically fermented bamboo flaky soil from the Science and Technology Park at Walailak University, Thailand, was sterilized by autoclaving at 121 °C and 15 psi for two 30 min cycles with a 24 h interval between each cycle. Each planting hole in the sterilized soil bed received 0.1 mL of the prepared *P. aphanidermatum* inoculum. Seeds of King Cup Peach Tomato TA072 (known to be susceptible to damping-off) were pre-soaked in sterile distilled water for 1 h for surface sterilization and then planted in the inoculated soil. Seedlings were monitored for disease symptoms (pre-emergence damping-off: seeds failing to germinate; post-emergence damping-off: water-soaked lesions at the stem base) for a period of 7 days post-seeding. Disease severity was assessed by recording the number of tomato plants exhibiting symptoms. The disease incidence percentage was calculated using the following formula: Disease Incidence (%) = (Nd/Nt) × 100, where Nd represents the number of diseased tomato plants and Nt represents the total number of tomatoes planted [28,29].

2.3. Interaction between Trichoderma asperellum Strains

The compatibility of two *Trichoderma asperellum* strains, *T. asperellum* NST-009 (the native Nakhon Si Thammarat strain, Walailak University, Thailand) and *T. asperellum* CB-Pin-01 (the commercial Thai strain, Kasetsart University, Thailand) [14–18], was evaluated using a dual culture assay. Both strains were grown on PDA medium for 3 days at 28 ± 2 °C. Using a sterile cork borer (diameter 0.5 cm), actively growing mycelial plugs were harvested from each strain. Fresh PDA plates were prepared. On each plate, one plug from each *Trichoderma* strain was placed on opposite sides, approximately 5 cm apart. This configuration allowed for the observation of interactions between the colonies. Plates were incubated at 28 ± 2 °C, and after a designated incubation period (7 days), the following aspects were examined to assess compatibility: (1) Formation of clear zones: Clear zones around the mycelial plugs would indicate inhibition, suggesting antagonism between the strains. (2) Colony interaction lines: The development of distinct lines between the growing colonies would signify interactions and provide insights into their compatibility.

2.4. Efficacy Study of Trichoderma asperellum Strains and Metalaxyl against Pythium aphanidermatum In Vitro

This section describes an experiment designed to evaluate the effectiveness of different methods in controlling *P. aphanidermatum*, a fungal plant pathogen, under laboratory conditions (in vitro). A Completely Randomized Design (CRD) was employed with four treatments and five replications/treatments. The treatments include T1: *Trichoderma asperel-*

lum strain NST-009, T2: *Trichoderma asperellum* strain CB-Pin-01, T3: Metalaxyl (chemical fungicide), and T4: Control (only *P. aphanidermatum*). *P. aphanidermatum* was grown on Potato Dextrose Agar (PDA) for three days at 28 ± 2 °C. A sterile cork borer (diameter: 0.5 cm) was used to obtain a plug of actively growing *Pythium* sp. mycelium from the colony edge. The *P. aphanidermatum* plug was placed on a fresh PDA plate along with the corresponding treatment, Metalaxyl. The fungicide was incorporated into the PDA media using the spread plate technique (0.1 mL per plate) at a concentration of 1 g L^{-1} water. *Trichoderma asperellum* strains: a dual culture technique was used. A separate PDA plate was inoculated with both the *P. aphanidermatum* plug and a 0.5 cm diameter plug of the respective *T. asperellum* strain (NST-009 or CB-Pin-01). Control: the *P. aphanidermatum* plug was placed on a plain PDA plate without any treatment. All plates were incubated at room temperature. The experiment was terminated when the *P. aphanidermatum* colony on the control plates completely covered the dish. The diameter of the *P. aphanidermatum* colony on each treatment plate was measured.

The inhibition percentage of *T. asperellum* to inhibit the mycelial growth of *P. aphanidermatum* was calculated by a formula as follows: [(Rc − Rt)/Rc × 100] when Rc was the mean of mycelial radius of *P. aphanidermatum* in the control and Rt was the mean of mycelial radius of *P. aphanidermatum* in the dual culture test in Petri dish.

The overgrowth rate of *T. asperellum* to cover the mycelia of *P. aphanidermatum* was calculated by a formula as follows: (C1 − C2)/T when C1 was the mean of the mycelial radius of *T. asperellum* at the day of study (7 d after incubation), C2 was the mean of the mycelial radius of *T. asperellum* at the initial day that *P. aphanidermatum* was attacked by *T. asperellum*, and T was the time in a unit day between C1 and C2.

2.5. Scanning Electron Microscopy Assay

This step aims to investigate spore formation and potential physical destruction of *Pythium* sp. filaments by *T. asperellum* NST-009. Fungal filaments obtained from the *T. asperellum* NST-009 and *P. aphanidermatum* dual cultures after 3 days were observed using a field emission scanning electron microscope (FE-SEM) (Merlin Compact; Zeiss, Jena, Germany), an energy dispersive X-ray spectrometer (Aztec; Oxford Instruments, Abingdon, Oxfordshire, UK), and electron backscatter diffraction (Nordlys Max; Oxford Instruments, Abingdon, Oxfordshire, UK). After a colony of *P. aphanidermatum* was attacked by the mycelia of *T. asperellum*, the samples of the activity zone were cut into small pieces (0.5 cm × 0.5 cm), fixed in 2.5% glutaraldehyde at 4 °C for 24 h, and rinsed with distilled water before dehydration in a 30–100% alcohol series. The samples were dried in a critical point dryer machine, K850 (Quorum, Laughton, East Sussex, UK), followed by gold coating using a Sputter Coater 108 (Cressington, Watford, UK). The coated samples were then immediately examined by FE-SEM [23].

2.6. Efficacy Studies in Controlling Plant Pathogenic Fungi at the Greenhouse Level

This study evaluates the effectiveness of various treatments in controlling fungi that cause plant diseases in a greenhouse setting. The experiment follows a Completely Randomized Design (CRD) with seven treatments, six replicated per treatment, involving ten plants per replicate. The treatments included T1: *T. asperellum* NST-009, T2: *T. asperellum* CB-Pin-01, T3: *T. asperellum* NST-009 + CB-Pin-01, T4: *T. asperellum* NST-009 + CB-Pin-01 + plant immune stimulant ($CaCO_3$; used 1.0 g L^{-1}), T5: Control (Metalaxyl), T6: Control 1 (only *P. aphanidermatum*), and T7: Control 2 (without *P. aphanidermatum*).

2.6.1. Preparation of Trichoderma Spore Powder

Trichoderma asperellum spores were cultivated on cooked rice using the following method: Cook rice at a ratio of 3 parts rice to 2 parts water. Place 300 g of cooked rice into a heat-resistant plastic bag (9 inches × 14 inches) and allow it to cool. Spray 1 mL of *Trichoderma* inoculum onto the semi-cooked rice. Seal the bag with a stapler and shake it. Puncture the bag 20–30 times with a needle, and then incubate for 7 days at 28 ± 2 °C. To

obtain concentrated spore suspensions, sift the fresh culture to separate the grains, resulting in powdered *Trichoderma* fungi.

2.6.2. Preparation of Tomato Seeds

The King Cup tomato variety TA072 seeds were treated as follows: Treatment 1–4: Soak seeds in a suspension of *T. asperellum* spores (50 g of *Trichoderma* powder mixed with 20 L of water) for 1 h. Treatment 5: Soak seeds in a Metalaxyl chemical solution for 1 h (20 g per 20 L of water). Treatment 6–7: Soak seeds in autoclaved water for 1 h.

2.6.3. Preparation of Pathogen Inoculum

Pythium aphanidermatum was cultured on PDA medium at 28 ± 2 °C for 5 days. The culture was then cut into pieces (0.5 cm × 0.5 cm) and mixed with autoclaved water at a rate of 1 culture dish per 500 mL. Each plant bed hole containing sterile potting soil received 0.1 mL of this *P. aphanidermatum* suspension. Prepared tomato seeds were then planted, with one seed per hole and 60 seeds per treatment. For treatment 7 (Control 2), no *Pythium* suspension was added.

2.6.4. Disease Severity Index, Root Colonization, and Trichoderma Population Assessment

After planting tomato seeds for 21 days, the disease incidence percentage was determined using the formula as follows: Disease Incidence (%) = (Nd/Nt) × 100, where Nd is the number of diseased plants. Nt is the total number of plants. Root colonization of *T. asperellum* was observed after the application of *T. asperellum* for 21 days. The tomato seedling roots were cut to a size of 1 cm in length, soaked in a 0.53% solution of sodium hypochlorite for 5 min, and washed with sterile water three times. The pieces of tomato root were then dried with sterile paper and put on Martin's medium (KH_2PO_4 1.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, peptone 5.0 g, dextrose 10.0 g, rose bengal 0.033 g, and agar 15.0 g, dissolved in 1000 mL distilled water and supplemented with 100 mg streptomycin). The experiment was designed in CRD with four replications per treatment. The colonization percentage of tomato seedling root was evaluated after incubation at 28 ± 2 °C for 4 days using the following formula: $R_C/R_T \times 100$, where R_C was the number of root pieces colonized by *T. asperellum* and R_T was the total number of root pieces [11,21]. The population of *Trichoderma* strains was evaluated using the dilution plate technique after the application of *T. asperellum* for 21 days. A total of 10 g of planting medium was added to a 250 mL flask containing 90 mL of sterile water and mixed using a shaker at 120 rpm for 30 min. The soil suspension was then diluted with sterile water at 10^{-1}- to 10^{-5}-fold, and 0.1 mL of diluted soil solution was dropped onto the surface of Martin's medium with a micropipette and spread over the soil solution with a sterile glass rod. The experiment was designed in CRD with four replications per treatment. The number of *Trichoderma* strains was counted after incubation at 28 ± 2 °C for 4 days [22,24].

2.7. Data Analysis

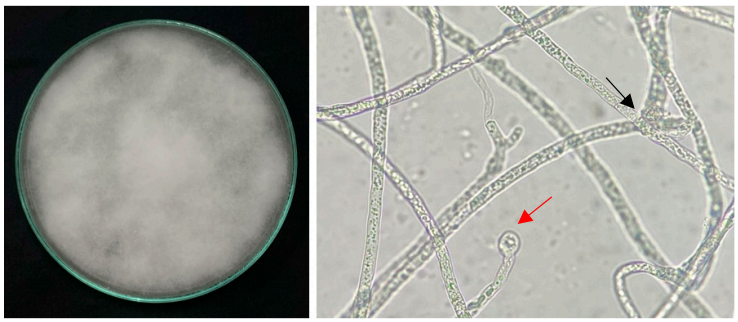
All the data were subjected to an analysis of variance (ANOVA), followed by a comparison using Duncan's multiple range test. The significance level was set at $p \leq 0.05$.

3. Results

3.1. Isolation and Identification of the Tomato Damping-Off Pathogen

Fungal isolation was performed using a tissue transplanting technique from symptomatic tomato root sections exhibiting signs of damping-off. The fungal colony characteristics are white, fluffy colonies on PDA with hypha growth patterns that emerged from the diseased tissue after incubation (Figure 1a). Microscopic examination (400× magnification) revealed the presence of propagative structures consistent with *Pythium* species, including sporangia and oospores (Figure 1b). Furthermore, the field emission scanning electron microscope (FE-SEM) analysis confirmed the presence of hyphae with characteristics typical of *Pythium* species. Based on morphological features observed under light

microscopy and FE-SEM, including the presence of non-septate, branched hyphae and the formation of oogonia and sporangia, the isolated fungal pathogen was tentatively identified as belonging to *Pythium aphanidermatum*. This identification was further supported by comparisons with previously published descriptions, such as those of Soliman et al. [5] and Al-Shuaibi et al. [30].



(**a**) (**b**)

Figure 1. The colony characteristics of *Pythium aphanidermatum* on PDA plate (**a**), hypha, sporangia (black arrow), and oospores (red arrow) characteristics of *P. aphanidermatum* under microscopy (400× magnification) (**b**).

3.2. Pathogenicity of Isolated Pythium Species

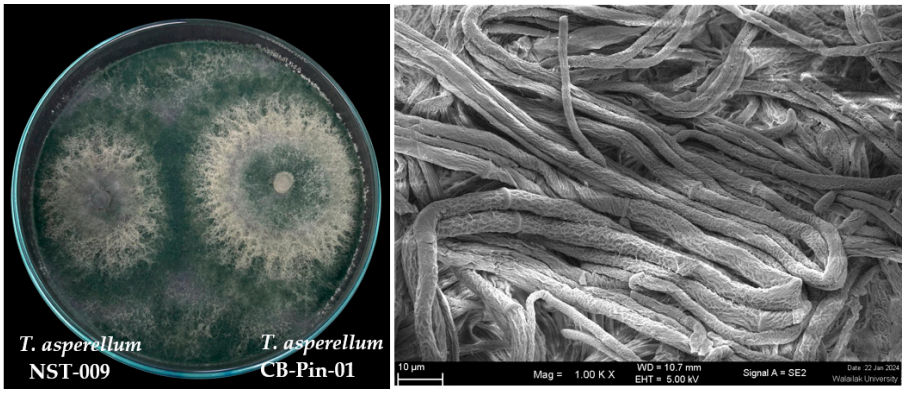
The pathogenicity of the isolated *Pythium aphanidermatum* was assessed through a greenhouse assay on tomato seedlings. Seven-day-old tomato seedlings (King Cup Peach Tomato TA072) exhibited various damping-off symptoms following inoculation with the fungal isolate. These symptoms included: (1) Pre-emergence damping-off, when seed rot occurred before germination; (2) post-emergence damping-off, when seedlings emerged but displayed stem rot at the soil line, characterized by water-soaked lesions, browning, and wilting; and (3) seedling death, when infected seedlings became stunted, discolored, and eventually died due to compromised root systems (Figure 2). The disease incidence was quantified by calculating the disease incidence percentage, which reached 63% in the inoculated group compared to the control treatment with autoclaved water. Moreover, the symptomatic tomato seedlings were re-isolated for the *Pythium* pathogen, confirming its presence through morphological characteristics. This re-isolation fulfills Koch's postulates, which state that to definitively identify a pathogen, the microbe must be consistently isolated from diseased tissue, grown in pure culture, and then reintroduced to cause the same disease in healthy plants.

(**a**) (**b**)

Figure 2. Comparison between normal seedlings in the control group (**a**) and tomato seedlings exhibiting typical signs of damping-off disease 7 days after seeding. The disease is characterized by dark lesions and constrictions on the stem near the soil line, often leading to stem collapse and seedling death (red arrow) (**b**).

3.3. Compatibility of Trichoderma asperellum Strains

The compatibility of the two *Trichoderma asperellum* strains, *T. asperellum* NST-009 and *T. asperellum* CB-Pin-01, was evaluated using a dual culture assay on potato dextrose agar (PDA) medium. Both strains exhibited growth toward each other on the same culture plate without the formation of clear inhibition zones, which typically indicate compatibility (Figure 3a). Additionally, examination under field emission scanning electron microscopy (FE-SEM) did not reveal any distinct lines of demarcation or inhibition points between the two fungal colonies (Figure 3b).



(**a**) (**b**)

Figure 3. Growth characteristics of 2 strains of antagonist fungi, *T. asperellum*, on PDA culture medium (**a**) and growth characteristics of 2 strains of *T. asperellum* antagonist filaments under field emission scanning electron microscopy (FE-SEM) (**b**).

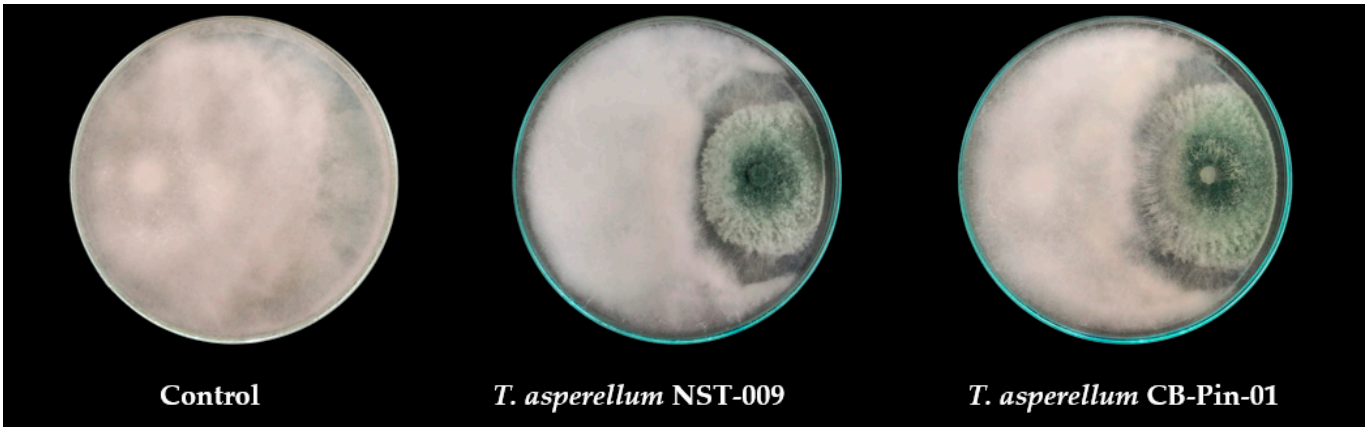
3.4. Efficacy in Controlling Plant Pathogenic Fungi at the Laboratory Level

Both *Trichoderma asperellum strains*, NST-009 and CB-Pin-01, significantly inhibited the growth of *Pythium aphanidermatum* compared to the control group (0% inhibition) (Table 1). These findings suggest their potential as biological control agents against this fungal pathogen. Notably, *T. asperellum* CB-Pin-01 displayed a slightly higher percentage of growth inhibition (89.27%) compared to *T. asperellum* NST-009 (84.52%) (Figure 4). While the fungicide Metalaxyl achieved complete inhibition (100%) of *P. aphanidermatum* growth (Figure 5), both *T. asperellum* strains exhibited positive overgrowth values (NST-009: 0.68 cm day^{-1}; CB-Pin-01: 0.73 cm day^{-1}). This indicates their potential to outcompete and suppress the pathogen on agar plates. Field emission scanning electron microscopy (FE-SEM) analysis revealed that *T. asperellum* NST-009 parasitized *P. aphanidermatum* mycelia, significantly altering their morphology. Compared to the control, treated hyphae exhibited pronounced wrinkling, rupture, and coiling by *T. asperellum* hyphae, indicative of parasitic interactions. Additionally, structural damage and abnormalities within *P. aphanidermatum* hyphae suggested antagonistic effects of *T. asperellum* (Figure 6).

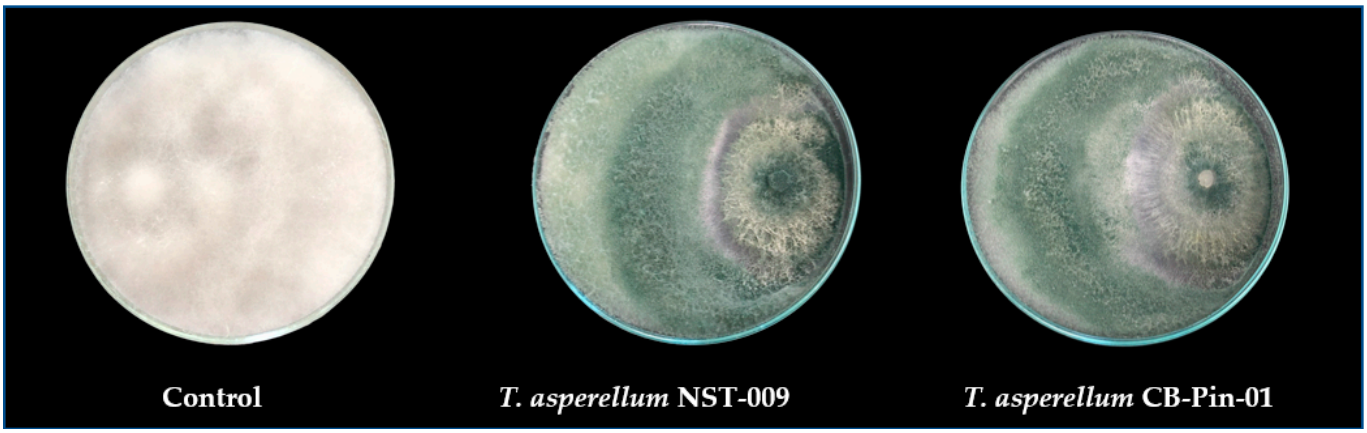
Table 1. The efficiency of *Trichoderma asperellum* strains and Metalaxyl chemicals to inhibit and overgrow the mycelia of *Pythium aphanidermatum* on potato dextrose agar after incubation at room temperature for 7 days.

Treatments	Mycelial Growth Inhibition (%)	Over Mycelial Growth (cm day^{-1})
T. asperellum NST-009	84.52 ^{c}	0.68 ^{a}
T. asperellum CB-Pin-01	89.27 ^{b}	0.73 ^{b}
Metalaxyl	100.00 ^{a}	-
Control	0.00 ^{d}	-
C.V. (%)	2.44	2.20

Mean values within the same columns followed by the same letter are not significantly different according to Duncan's multiple range test ($p < 0.05$).

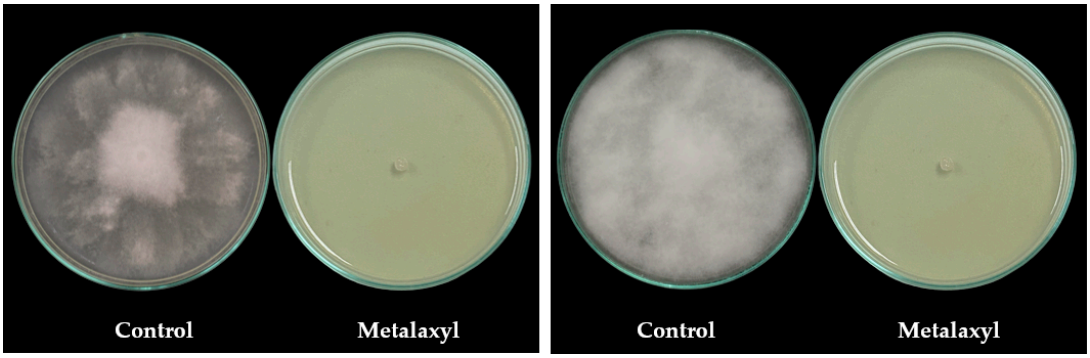


(**a**)



(**b**)

Figure 4. Efficacy in controlling *Pythium aphanidermatum* with *Trichoderma* on a PDA plate 3 days after testing (**a**) and its effectiveness in controlling *P. aphanidermatum* with *Trichoderma* on a PDA plate 7 days after the test (**b**).



(**a**) (**b**)

Figure 5. Efficacy in controlling *Pythium aphanidermatum* with Metalaxyl chemicals on PDA plates 3 days after test (**a**) and effectiveness in controlling *P. aphanidermatum* with Metalaxyl chemicals on PDA plates 7 days after testing (**b**).

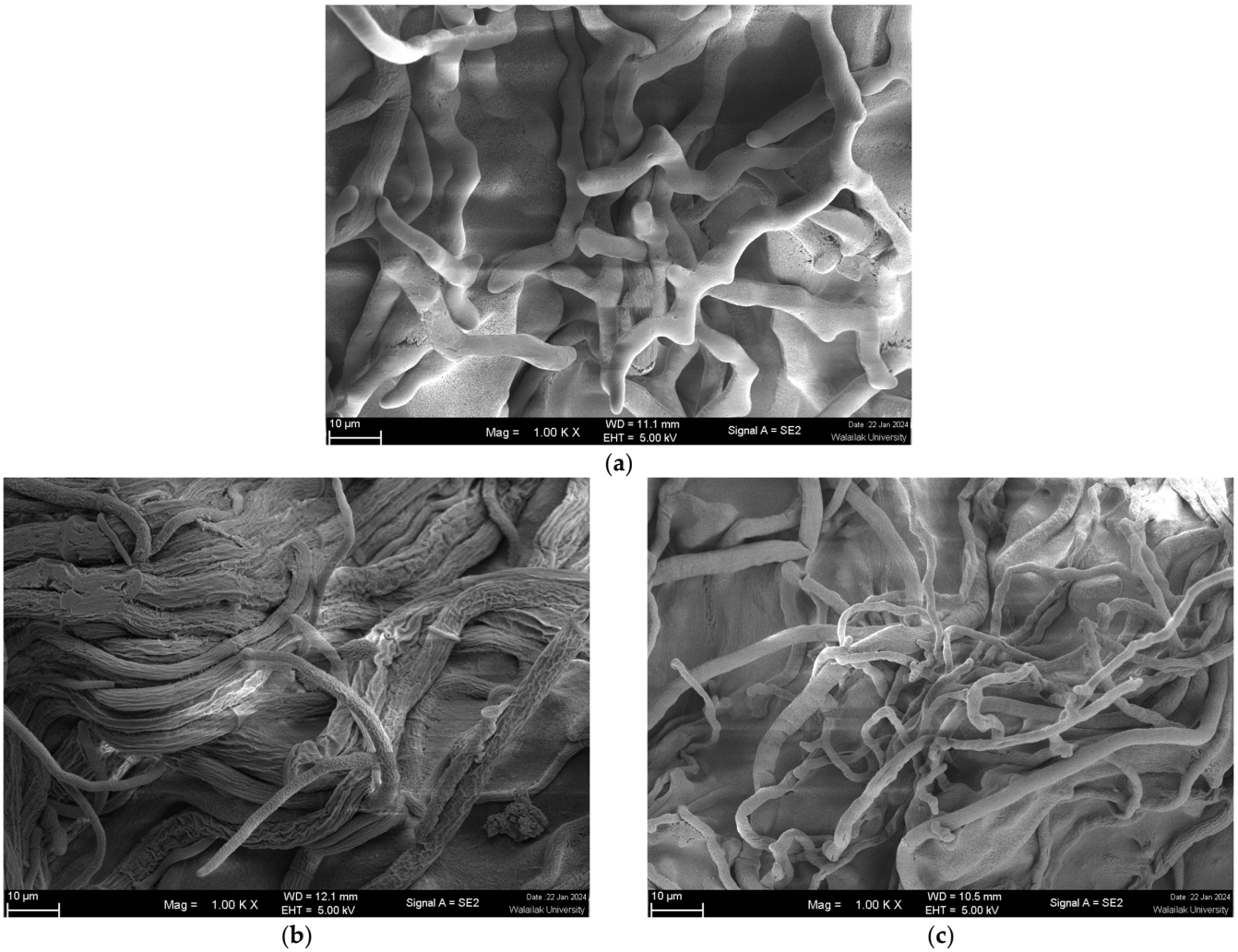


Figure 6. Hyphae of *P. aphanidermatum* (**a**), mycelia of *T. asperellum* NST-009 (**b**), and the effect of *T. asperellum* (T) on *P. aphanidermatum* (P) hyphae (**c**) under FE-SEM.

3.5. Effectiveness in Controlling Plant Disease Causative Fungi at the Greenhouse Level

3.5.1. Disease Incidence Percentage

Both single strains (NST-009 and CB-Pin-01) of *Trichoderma asperellum* significantly reduced disease incidence compared to plants exposed only to the *Pythium* fungus (causing the disease). Notably, combining the strains (*T. asperellum* NST-009 + *T. asperellum* CB-Pin-01) offered even greater protection, resulting in a disease incidence of just 20.55%. The addition of calcium carbonate ($CaCO_3$) as a plant immune stimulant further enhanced the effectiveness of the combined *Trichoderma* strains. This treatment (*T. asperellum* NST-009 + *T. asperellum* CB-Pin-01 + $CaCO_3$) achieved the lowest disease incidence of 17.78%. While the fungicide Metalaxyl also reduced disease incidence, it completely eliminated the beneficial *Trichoderma* population in the soil. This highlights the potential advantage of using *Trichoderma* as a more sustainable and long-term solution for managing damping-off disease, especially when combined with methods that boost plant immunity. The control groups serve as important references. Control 1 (with only *P. aphanidermatum*) had the highest disease incidence (66.55%), showcasing the destructive impact of the pathogen. Control 2 (without *P. aphanidermatum*) with no disease presence (0% disease incidence) confirms the absence of the disease in healthy plants (Table 2).

Table 2. The tomato damping-off disease severity index, root colonization, and *Trichoderma* population in the soil after testing for 21 days in greenhouse conditions.

Treatments	Disease Incidence (%)	Root Colonization (%)	*Trichoderma* Population (CFU Soil 1 g^{-1})
T. asperellum NST-009	35.55 ^{c}	91.67 ^{ab}	5.17×10^{7} ^{bc}
T. asperellum CB-Pin-01	41.67 ^{b}	87.50 ^{b}	4.93×10^{7} ^{c}
T. asperellum NST-009 + *T. asperellum* CB-Pin-01	20.55 ^{d}	100.00 ^{a}	5.83×10^{7} ^{ab}
T. asperellum NST-009 + *T. asperellum* CB-Pin-01+ plant immune stimulant ($CaCO_3$)	17.78 ^{d}	100.00 ^{a}	6.17×10^{7} ^{a}
Metalaxyl	22.78 ^{d}	0.00 ^{c}	0.00 ^{d}
Control 1 (only *P. aphanidermatum*)	66.55 ^{a}	0.00 ^{c}	0.00 ^{d}
Control 2 (without *P. aphanidermatum*)	0.00 ^{e}	0.00 ^{c}	0.00 ^{d}
C.V. (%)	9.06	9.93	12.44

Mean values within the same columns followed by the same letter are not significantly different according to Duncan's multiple range test ($p < 0.05$).

3.5.2. Root Colonization

Both single strains of *Trichoderma asperellum* (NST-009 and CB-Pin-01) significantly increased root colonization compared to the control groups (0% colonization). This indicates that these beneficial fungi can establish themselves within the root system, potentially enhancing plant growth and providing protection against pathogens. The combination of *T. asperellum* NST-009 and *T. asperellum* CB-Pin-01 (100% colonization) achieved the highest root colonization rate compared to the single strains (around 91%). This suggests a potential synergistic effect between the strains, leading to a more robust colonization of the root system. The addition of calcium carbonate ($CaCO_3$) as a plant immune stimulant did not appear to significantly affect root colonization compared to the treatment with just the combined *Trichoderma* strains (both at 100%). The fungicide Metalaxyl completely suppressed root colonization (0%), as expected. While it can be effective against fungal pathogens, it also eliminates beneficial microbes like *Trichoderma* that can contribute to plant health. As expected, the control groups with or without the *Pythium* fungus (causing damping-off disease) showed no root colonization by beneficial fungi (0%) (Table 2).

3.5.3. Trichoderma Population

All treatments containing *Trichoderma asperellum* strains (either alone or combined) had a population of *Trichoderma* fungi in the soil, while the control groups had none. This confirms the presence and establishment of these beneficial fungi in the treated soils. Interestingly, the combined application of *T. asperellum* NST-009 and CB-Pin-01 (5.83×10^{7} CFU g^{-1}) did not result in the highest *Trichoderma* population. The treatment with only *T. asperellum* NST-009 (5.17×10^{7} CFU g^{-1}) had a similar population, while the treatment with $CaCO_3$ and both strains (6.17×10^{7} CFU g^{-1}) showed the highest population. The treatment with the plant immune stimulant $CaCO_3$ and both *Trichoderma* strains had the highest *Trichoderma* population (6.17×10^{7} CFU g^{-1}). While not statistically significant compared to the treatment with just the combined strains based on the letter groupings, this suggests a possible positive influence of $CaCO_3$ on *Trichoderma* growth in the soil. As expected, the fungicide Metalaxyl completely eliminated the *Trichoderma* population (0 CFU g^{-1}) in the soil. This highlights the potential drawback of fungicides, as they can target both beneficial and harmful fungi (Table 2).

4. Discussion

In this study, we explored the integration of two *Trichoderma asperellum* strains (NST-009 and CB-Pin-01) with calcium carbonate ($CaCO_3$) to enhance plant immune responses against damping-off disease in tomato seedlings caused by *P. aphanidermatum*. The *Pythium* pathogen was accurately identified using a combination of classical and advanced techniques. Initially, tissue transplanting of infected tomato roots onto potato dextrose agar

(PDA) allowed for the isolation of the pathogen. The colonies displayed white, fluffy, and fibrous characteristics consistent with *Pythium* species. Microscopic examination at 400× magnification confirmed the presence of diagnostic *Pythium* structures, including sporangia and oospores. Further characterization using field emission scanning electron microscopy (FE-SEM) revealed mycelial morphology typical of *P. aphanidermatum*. These findings align with previous reports by Soliman et al. [5] and Al-Shuaibi et al. [30] describing the characteristic morphological features of *P. aphanidermatum*, such as branched, filamentous, and non-septate hyphae, as well as spherically or pear-shaped oogonia. This multi-method approach provided robust evidence for the identification of *P. aphanidermatum* as the causative agent of damping-off disease in our tomato seedlings. However, confirmation with molecular techniques such as sequencing of the internal transcribed spacer (ITS) region would be beneficial and will be incorporated as part of future research. There are several reports that *Pythium* species, including *P. aphanidermatum*, *P. ultimum*, *P. indicum*, and *P. debaryanum*, caused the damping-off disease in tomatoes [2,7,31,32]. However, *P. aphanidermatum* is the most important tomato damping-off causal agent in Thailand.

To further validate the pathogenicity of the isolated *Pythium* sp., we conducted a greenhouse assay. Seven-day-old King Cup Peach Tomato TA072 seedlings were inoculated with the pathogen and monitored for disease symptoms. The infected seedlings exhibited classic damping-off symptoms, including both pre-emergence seed rot and post-emergence stem rot, leading to water-soaked lesions, browning, wilting, and eventual mortality due to root system damage. The disease incidence percentage of the inoculated group was calculated at 63%, significantly higher than the control group, which was treated with sterile water. This high disease incidence percentage confirms the pathogenic potential of the isolated *P. aphanidermatum* to cause damping-off disease in tomato seedlings [4,8].

This study also aimed to assess the compatibility of the two *Trichoderma asperellum* strains (NST-009 and CB-Pin-01) for potential co-application. Using a dual culture assay on PDA, both strains demonstrated mutual tolerance and an absence of antagonistic interaction, as evidenced by their growth towards each other without forming inhibition zones. This compatibility was further validated using FE-SEM, which provided high-resolution images showing no distinct demarcation or inhibition points between the colonies. The absence of these physical boundaries supports the idea that NST-009 and CB-Pin-01 can coexist without hindering each other's growth. Such compatibility is crucial for their combined use in biocontrol applications, as it suggests they could work synergistically rather than competitively.

In evaluating the mycoparasitic capabilities of the *Trichoderma asperellum* strains against *P. aphanidermatum*, both NST-009 and CB-Pin-01 exhibited substantial mycoparasitic activity. In controlled laboratory conditions, CB-Pin-01 showed a slightly higher inhibition rate (89.27%) compared to NST-009 (84.52%), while the fungicide Metalaxyl achieved complete inhibition (100%). Despite the efficacy of fungicides like Metalaxyl, they pose the risk of disrupting soil microbiomes by eliminating both pathogenic and beneficial microbes. Conversely, the *Trichoderma* strains not only inhibited pathogen growth but also demonstrated positive overgrowth rates (0.68 cm day^{-1} for NST-009 and 0.73 cm day^{-1} for CB-Pin-01), suggesting their ability to outcompete *P. aphanidermatum* through space and nutrient competition. These findings underscore the potential of NST-009 and CB-Pin-01 as biocontrol agents that can effectively suppress *P. aphanidermatum* while potentially maintaining or enhancing soil health. Regarding mycoparasitism, several studies have investigated the use of *Trichoderma* strains to inhibit *P. aphanidermatum*. For example, Khare et al. [33] conducted in vitro screenings of wild-type and mutant strains of *T. viride* 1433 against *P. aphanidermatum* using the dual culture method. They observed inhibition of the linear growth of *P. aphanidermatum* due to the production of volatile and non-volatile metabolites. Moreover, Kamala et al. [34] found that out of the total *Trichoderma* isolates tested, 32% exhibited antifungal antagonistic activity in vitro against *P. aphanidermatum*, the causal agent of damping-off disease in beans. Six isolates significantly inhibited the external growth of *P. aphanidermatum*, with inhibition percentages ranging from 4.16% to 84%. The

maximum inhibition was exhibited in T105 (84%), while the lowest was observed in T71 (4.16%). In addition, Singh et al. [35] evaluated the effect of four *Trichoderma* isolates—*T. harzianum* (Th Azad), *T. viride* (01PP), *T. asperellum* (Tasp/CSAU), and *T. longibrachiatum* (21PP)—against *P. aphanidermatum* in vitro. *T. harzianum* (Th. Azad) recorded the maximum growth inhibition (60.38%) against *P. aphanidermatum* and produced higher amounts of volatile and non-volatile metabolites. Furthermore, Al-Shuaibi et al. [35] reported that *T. ghanense* (T1) achieved an inhibition rate of 44.6% against *P. aphanidermatum*, while *T. citrinoviride* (T2) suppressed the mycelial growth of *P. aphanidermatum* by 31.3%. These studies highlight the potential of various *Trichoderma* strains in the biological control of *P. aphanidermatum*, showcasing their varying degrees of effectiveness and mechanisms of action.

The combined application of the *Trichoderma asperellum* strains with $CaCO_3$ was assessed for controlling damping-off disease. Individually, both strains significantly reduced disease severity compared to the untreated, *Pythium*-infected plants, with the combined application resulting in even lower disease incidence (20.55%). The addition of $CaCO_3$ further enhanced this effect, reducing the disease incidence to 17.78%. Although the fungicide Metalaxyl also effectively reduced disease incidence, it completely eradicated *Trichoderma* from the soil, highlighting the long-term benefits of using *Trichoderma* for sustainable disease management. Control groups confirmed the experimental setup's validity, with the *Pythium*-infected control showing the highest disease incidence (66.55%) and the healthy control showing no disease presence. These results suggest that *Trichoderma* strains, particularly when used together and with $CaCO_3$, offer a sustainable and effective approach to managing damping-off disease.

Further analysis focused on the ability of the *Trichoderma* strains to colonize tomato roots. Both NST-009 and CB-Pin-01 individually showed significant root colonization compared to control groups with no *Trichoderma* colonization. The combined strains resulted in the highest root colonization rate (100%), indicating a synergistic effect that enhances colonization efficiency. Interestingly, the addition of $CaCO_3$ did not significantly alter the colonization rate compared to the combined *Trichoderma* treatment alone, suggesting that $CaCO_3$ does not directly influence *Trichoderma* colonization under the tested conditions. As expected, Metalaxyl treatment completely suppressed *Trichoderma* colonization, reaffirming its broad-spectrum fungicidal activity. These findings highlight the effective colonization capacity of *Trichoderma asperellum* strains and the potential for enhanced colonization when used in combination.

This study also evaluated the establishment and population dynamics of *Trichoderma asperellum* in the soil. All treatments with *Trichoderma* strains showed successful establishment in the soil, as evidenced by significant fungal populations. The combined application of NST-009 and CB-Pin-01 resulted in a notable population (5.83×10^7 CFU g^{-1}), although not the highest observed. The treatment with only NST-009 yielded a similar population, indicating that both strains can establish themselves effectively in the soil environment. The inclusion of $CaCO_3$ alongside both strains led to the highest *Trichoderma* population (6.17×10^7 CFU g^{-1}), suggesting a potential positive influence of $CaCO_3$ on *Trichoderma* growth, although the increase was not statistically significant. In contrast, Metalaxyl completely eradicated the *Trichoderma* population, highlighting the fungicide's disruptive impact on beneficial soil microorganisms. These observations suggest that while *Trichoderma asperellum* strains can effectively establish themselves in the soil, further research is needed to understand the potential benefits of $CaCO_3$ in supporting their growth.

Calcium carbonate ($CaCO_3$), commonly recognized as limestone or chalk, is currently being explored for its potential to enhance plant immunity and plant disease control [20,21]. The exact mechanisms by which $CaCO_3$ influences plant defense responses are not yet fully understood. However, there is evidence suggesting that $CaCO_3$ can fortify the cell walls of tomato seedlings and may indirectly promote plant health by encouraging the growth and activity of *Trichoderma* spp., a beneficial biocontrol fungus. Although initial studies on the direct immune-stimulating effects of $CaCO_3$ are inconclusive, recent research

has indicated a possible increase in *Trichoderma* populations with $CaCO_3$ application. This finding, even though not statistically significant, hints at a potential indirect pathway for improving plant disease resistance. *Trichoderma* is known for its diverse mechanisms for suppressing plant pathogens, and the observed increase in its population may be a promising sign of $CaCO_3$'s role in supporting biocontrol efforts. The mechanisms through which $CaCO_3$ might enhance *Trichoderma* growth include its ability to buffer soil pH, creating an alkaline environment conducive to *Trichoderma*'s proliferation, and its influence on nutrient availability, which could give *Trichoderma* a competitive edge over pathogens. Despite these promising observations, more research is essential to validate these interactions and understand the complex dynamics between $CaCO_3$ and *Trichoderma*. Further large-scale field studies are needed to confirm the observed increase in *Trichoderma* populations and assess its impact on disease control. These studies should investigate various types and concentrations of $CaCO_3$ and their effects on different plant species to thoroughly evaluate $CaCO_3$'s potential as an enhancer of *Trichoderma*-mediated biocontrol. This research could lead to more sustainable strategies for managing crop diseases (Figure 7).

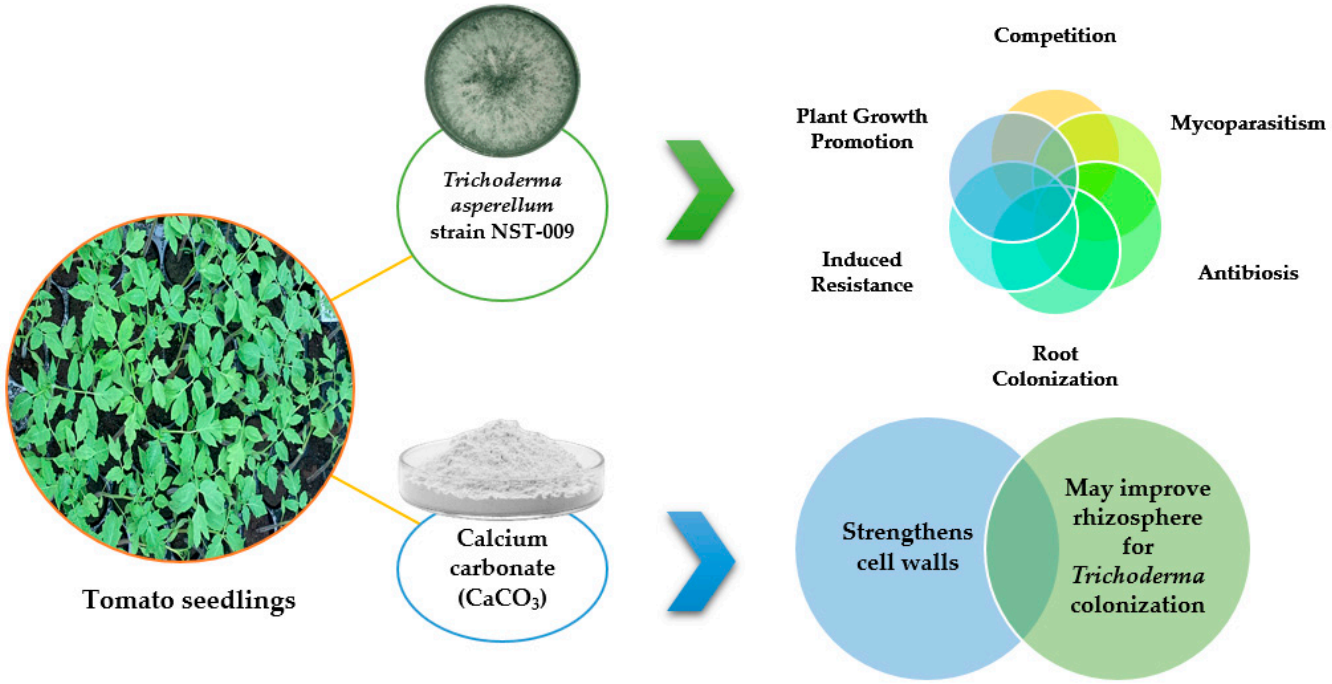


Figure 7. The potential of combining *Trichoderma asperellum* with calcium carbonate ($CaCO_3$) as a plant immune stimulant for controlling damping-off disease caused by *Pythium aphanidermatum* in tomato seedlings.

5. Conclusions

This study demonstrates the potential of combining two *Trichoderma asperellum* strains (NST-009 and CB-Pin-01) for the biocontrol of tomato damping-off disease caused by *P. aphanidermatum*. The addition of calcium carbonate may further enhance disease control, although the underlying mechanisms require further investigation. This approach offers a promising and sustainable alternative to chemical fungicides for managing damping-off disease in tomato production.

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