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Potential of *Trichoderma asperellum* as a bio-control agent against citrus diseases caused by *Penicillium digitatum* and *Colletotrichum gloeosporioides*

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Abstract *Colletotrichum gloeosporioides* is the main cause of citrus post-bloom and pre-harvest fruit drops, resulting in up to 65% and 22% of citrus crop loss, respectively, while *Penicillium digitatum* primarily causes green mold on postharvest citrus fruits accounting for 90% of total citrus fruit loss due to postharvest decay. Biocontrol agents are considered as eco-friendly and bio-safe alternatives of fungicides, and hence, being actively seeking. Previously, several *T. asperellum* strains have been demonstrated to be effectively inhibit either *P. digitatum* or *C. gloeosporioides* growths. We, therefore, wondered whether our *Trichoderma* isolates could simultaneously inactivate *P. digitatium* and *C. gloeosporioides in vitro* and protect citrus crops from green mold and anthracnose. Here, we defined three *T. asperellum* strains, which inhibited *P. digitatum* and *C. gloeosporioides* extension by around 99% and 77%, respectively. Single factor experiments showed that a medium containing 2% of sucrose and 1% of peptone on rice husks cultivated at 28^{o}C for 15 days was the best condition for these strains to produce conidia. Additionally, supplement of conidial suspension with 10% glycerol, 0.2% CMC, and 0.3% Tween 80 preserved spore viability by 80% after 2 months of storage. The development of citrus green mold and anthracnose was also inhibited in the presence of *T. asperellum* formulated conidia. Overall, these data indicated a potential application of the formulated conidia as a biocontrol agent in preventing citrus crop loss caused by both preharvest and postharvest diseases.

Keywords: Anthracnose, Bio-control, *Colletotrichum gloeosporioides*, Green mold, *Penicillium digitatum*, *Trichoderma asperellum*.

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Introduction

Citrus is the largest fruit crop, and commercially cultivated in about 140 countries all over the world (Ismail and Zhang, 2004). The citrus industry not only generates US$ billions of commodities, but also provides jobs to millions of workers worldwide. As in 2017, the global production of orange was 47.6 million metric tons and expected to increase to 51.8 million tons in 2018/2019 (citrus: world markets and trade). However, citrus diseases caused by both pre-harvest and post-harvest infections can result in an economic loss of up to 50% of the crop.

Pre-harvest diseases are the consequence of pathogen infection before fruit harvest while post-harvest decays can be the results of both pre- and post-harvest infection. The pre-harvest pathogens like *C. gloeosporioides* can damage the crop at a various stage of fruit development. For example, *C. gloeosporioides* causes post-bloom and preharvest fruit drops, leading to the crop loss of up to 65% and 22%, respectively (Naqvi, 2004). Under suitable condition, latent infection of *C. gloeosporioides* can become active and cause necrosis of leaves, flowers, and fruits, leading to a serious crop loss. Green mold, blue mold, and sour rot caused by *P. digitatum*, *Penicillium italicum, Galactomyces citri-aurantii*, respectively, are the most frequent diseases of harvested citrus fruits worldwide. Among them, postharvest green mold is the most prevalent, and can account for up to 90% of the total citrus fruit loss due to the postharvest decay (Zhu *et al*., 2017, Regnier *et al*., 2014).

C. gloeosporioides is of the top ten plant pathogens and can cause anthracnose, dieback, fruit and pod rot, and leaf spot diseases on a wide range of host. *C. gloeosporioides* damages the hosts via biotrophic and necrotrophic infections. In a biotrophic or latent phage, *C. gloeosporioides* penetrates the host cuticle and produces primary hyphae, which are quiescent and do no harm to the host. Under suitable conditions, *C. gloeosporioides* becomes active and start a necrotrophic phage by generating secondary hyphae, which spread and kill the host cells, leading to the formation of round dark sunken lesions. The lesions expand rapidly on the fruit skin and cause rot when infection gets into the pulp (Sharma and Kulshrestha, 2015). On PDA plates, *C. gloeosporioides* aerial mycelium forms a thick to sparse lawn with whitish to dark grey colors. Irregular acervuli contain light brown conidiophores, which appear orange and slimy in mass. Conidia are single and hyaline cells with cylindrical shapes and obtuse ends (Alemu, 2014).

P. digitatum is a necrotrophic fungus, which can only infect citrus fruits through mechanical injuries occurring during harvesting, handling, transporting, storing, and marketing processes. Under certain circumstances, *P.*

digitatum conidia on the wound surface of citrus fruits can germinate to produce germ tubes, which penetrate into the pericarp cells, extend into mesocarp cells, and gradually invade neighbor cells (Cheng *et al*., 2020). At 24^{o}C, the active infection takes place within 2 days and the white mycelium colonizes on the fruit surface within 3 days (Holmes and Eckert, 1999). As the white mycelia gradually spread and begin to produce conidia, the center of the mycelial mass turns olive green. Near the end of the disease cycle, the *P. digitatum* mycelium cover most, if not all, of the fruit surface and turn it into an empty, dry shell.

Currently, chemical fungicides are considered as the standard procedure for controlling citrus diseases in many citrus-producing areas. However, the use and misuse of synthetic fungicides in managing citrus diseases raised public concerns due to their risks for human health and environment. Hence, developing eco-friendly and bio-safe methods as alternatives of synthetic pesticides has been a priority. Biocontrol agents are safer for human health and environment than synthetic fungicides because they eliminate fungal pathogens based on the principle of predation and parasitism.

Trichoderma spp. is a genus of fungi present in all soils, and has been shown to protect plants from a variety of infectious pathogens, and to promote vegetative growth. *Trichoderma* spp. can be produced rapidly on multiple substrates at a low cost and in diverse industrial fermentation systems. The end-products i.e., pure spores, conidia suspensions, or liquid culture filtrates can be incorporated into various formulations, which can be stored for a long period without losing their efficacy. Because of these properties, dozens of *Trichoderma*-based products for crop protection, growth, and productivity have been commercialized worldwide. *Trichoderma* – based preparations can be formulated as (1) wettable powder such as Bio-Funguss, Binab Ts, Rootshields, Trichodexs, Mycobacs; (2) granular formulations i.e., Bio-Funguss, Rootshields, T-22 Planter Box, Trichoderma 2000s; (3) a pelleted formulation including Binab Ts, and; (4) a liquid formulation (conidial suspension) like Promotes. The formulations can be applied as foliar spray, pre-planting application, post-pruning treatment, incorporation in the soil during seeding or transplant, irrigation, and root dip or drench (Woo *et al*., 2014).

The efficacy of several *Trichoderma* strains on treating citrus diseases has also been documented. For example, Ferreira *et al.* (2020) showed that *T. harzianum, T. guizhouense, T. atroviride* and *T. koningiopsis* can effectively eliminate *P. digitatum, Alternaria alternata* and *C. gloeosporioides*, of which, the *T. harzianum* strain was the most effective for inactivating *P. digitatum in vivo*. This strain could also prevent rot formation in 80% of the fruits after 3 weeks of *P. digitatum* infection (Ferreira *et al*., 2020). Borrás and Aguilar

(1990) also reported that citrus fruits pre-treated with *Trichoderma viride* had no sign of *P. digitatum* development after 5 days of *P. digitatum* infection (Borrás and Aguilar, 1990). *T. viride* and *T. asperellum* has also been demonstrated to protect papaya fruits from *C. gloeosporioides*-caused rot (González-Estrada *et al*., 2018). In previous research, we collected twenty *Trichoderma* isolates from the orange orchard in Ha Giang, Vietnam, of which, ten exhibited at least 70% of inhibition radical growth of *P. digitatium*, *Fusarium oxysporum*, and *Phytophthora capsici* in agar diffusion assays. We, then, chose three isolates with the highest antagonistic activity to perform taxonomic analyses. Basing on morphological and molecular characteristics, the selected isolates named Tr.6, Tr.7, and Tr.8 were identified as members of *T. asperellum* species (Vu and Tran, 2020). Additionally, several strains of the *T. asperellum* species have also been demonstrated to inhibit *C. gloeosporioides* growth (Ramírez-Olier *et al*., 2019, Valenzuela *et al*., 2015). We, therefore, wondered whether our *Trichoderma* isolates could simultaneously inactivate *P. digitatium* and *C. gloeosporioides* and could protect citrus crops from green mold and anthracnose.

Thus, this study aimed to screen *Trichoderma* isolates from our collection to define strains with the strongest antagonism against *P. digitatium* and *C. gloeosporioides*, to optimize conditions at which selected strains generate the highest amount of conidia, to formulate *Trichoderma*-based preparation, and to evaluate the effectiveness of the formulation on protection of citrus from *P. digitatium*-caused green mold and *C. gloeosporioides*-caused anthracnose.

Materials and Methods

Fungal strains

P. digitatium HGP82, *C. gloeosporioides* HGC286, and 10 *Trichoderma* isolates including *T. asperellum* Tr.6, Tr.7, and Tr.8 strains were obtained from Center of Experimental Biology, National Center for Technological Progress, Ministry of Science and Technology, Vietnam.

Preparation of fungal spore suspension

For spore preparation, the fungal strains were grown on the PDA plates at 25-28^{o}C for 3-5 d. Sterile distilled water was added to the agar plate surface and spores were liberated from fungal mycelium by scraping with a sterile glass spreader. The liquid mixture was collected and filtered through Miracloth

(Calbiochem, Darmstadt, Germany) before a centrifugation at 4000 rpm for 10 min. The spore pellet was washed twice with sterile distilled water and resuspended in sterile distilled water again to obtain a final spore suspension. Fungal spore concentration was quantified under microscopy using a hemocytometer (HEINZ HERENZ Medizinalbedarf GmbH, Hamburg, Germany). The obtained spore suspension was adjusted to the concentration of 10^6 spores/ml. The spore suspension was directly used after preparation or stored at 4^oC for later use (Vu *et al*., 2018). Different carbon or nitrogen sources and concentrations were manipulated in PDA media to optimize culture conditions for conidial generation.

Antagonistic activities of Trichoderma isolates

The dual culture assays were conducted by inoculating 10 µl spore suspension (10^6 conidia/ml) of either *P. digitatium* HGP82, or *C. gloeosporioides* HGC286 with 10 µl spore suspension (10^6 conidia/ml) of each *Trichoderma* isolate at opposite sites on the same PDA plates (circle diagram in Fig. 1). The plates were incubated at 25 ± 2℃ for 7 days and observed periodically. Control plates were maintained without *Trichoderma* isolates. Each experiment was replicated three times. Percentage of growth inhibition was estimated by the formula of $I = (C-T)/C \times 100$, where C is mycelial growth of pathogens in control plate, T is mycelial growth of pathogens toward *Trichoderma* isolates and I is inhibitory percentage of mycelial growth (El_Komy *et al*., 2015).

In vivo antagonism tests against P. digitatum and C. gloeosporioides

To determine the preventive effects of the *T. asperellum* Tr.6, Tr.7, and Tr.8 strains on the development of green mold and anthracnose *in vivo*, freshly harvested citrus fruits or leaves were soaked into conidial suspension for 30 minutes before fungal infection. Ten fruits and leaves were used in each treatment and the experiment was repeated 3 times. The degree and incidence of fruit decay were observed at day 3, 7 and 10.

Fungal infections

Surfaces of citrus fruits and leaves were dis-infected with SDW and 70% ethanol, respectively. Wounds (10mm length and 2mm depth) on equatorial zones of orange fruits were scratched by sterile sticks while the ones on the leaves punched by sterile syringe needle. The wounds were inoculated

with either 10 µl of spores suspended in SDW (10^6 /mL) or SDW as mock control.

Preventive effects

Surfaces of freshly harvested citrus fruits and leaves were dis-infected with SDW and 70% ethanol, respectively. The fruits and leaves were soaked into conidial suspension for 30 minutes. After soaking, the fruits or leaves were drained, placed in honeycombed trays for 2 hours, and infected with either *P. digitatum* or *C. gloeosporioides* as described above. The fruits and leaves were then kept in sterile polyethylene boxes separately to prevent cross contamination and incubated for 10 days at 25^{o}C.

Conidial suspension

T. asperellum Tr.6, Tr.7, and Tr.8 strains were cultured on rice husks supplemented with 2% of sucrose and 1% of peptone at 28^{o}C for 15 days. The cultures were then soaked into SDW and filtered. The filtrates were supplemented with 10% glycerol, 0.2% CMC, and 0.3% Tween 80.

Results

T. asperellum Tr.6, Tr.7 and Tr.8 strains effectively inhibit *P. digitatum* and *C. gloeosporioides* growths *in vitro*

Identification of *Trichoderma* strains with the most antagonistic effects against *P. digitatium* and *C. gloeosporioides* would be critical for *Trichoderma* – based formulation. Fungal co-culture assays, hence, were conducted to define potential candidates. In agreement with our previous results, ten of our *Trichoderma* isolates were effectively against *P. digitatium* and *C. gloeosporioides* extension. Figure 1 exhibited the growth of ten *Trichoderma* strains co-cultured with either *P. digitatium* (upper panel) or *C. gloeosporioides* (bottom panel). All of ten *Trichoderma* isolates demonstrated inhibitory effects of at least 70% for both *P. digitatium* and *C. gloeosporioides*. Of these *Trichoderma* isolates, Tr.6, Tr.7, and Tr.8 strains displayed the most profound effects on *P. digitatum* and *C. gleosporioides* growths. These strains inhibited *P. digitatum* and *C. gleosporioides* extension by around 99% and 77%, respectively. The three *T. asperellum* Tr.6, Tr.7, and Tr.8 strains were, therefore, selected for further studies.

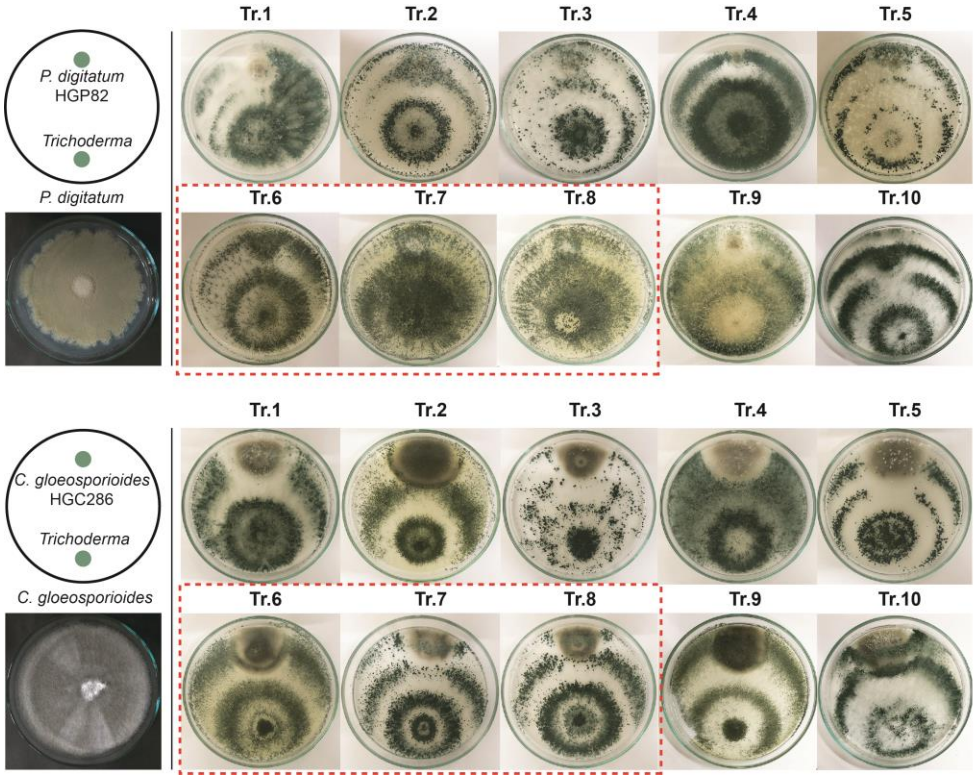

Figure 1. Antagonism of *Trichoderma* isolates against *P. digitatium* HGP82 strain (upper panel) and *C. gloeosporioides* HGC286 strain (lower panel) in fungal co-culture assays. Circular diagrams on the left demonstrated relative positions of fungal culture. Red boxes indicated three strains with the most inhibitory effects

Optimal conditions for conidial generations

Spores are the most resistant form in life cycles of microorganisms and usually used as bioweapons to combat plant diseases or to promote vegetative growth. Thus, optimal condition for maximizing conidial production will be essential for *Trichoderma* – based formulation. To optimize culture conditions for producing conidia of *T. asperellum* Tr.6, Tr.7, and Tr.8 strains, single factor experiments were performed. Conidial productions from 5 different carbon sources (Figure 2A) were compared. Anova analyses showed that *T. asperellum* Tr.6, Tr.7, and Tr.8 strains cultured on different carbon sources produced significant different amounts of spores. Sucrose had statistically higher effects on spore generation than lactose, maltose, and starch, but did not significantly differ from glucose despite slight increase (Figure 2A). Since sucrose

outperformed other carbon sources, its optimal concentration in media was carried out. As shown in Figure 2B, concentrations of conidia in fermented media with 2% of sucrose were about 30% higher than those containing 1% of sucrose. Number of spores produced in media with 3% or 4% of sucrose slightly decreased in comparison with those containing 2% of sucrose. Similar analyses showed that peptone significantly improved spore production for Tr.6, but was not significant for Tr.7 and Tr.8 despite slightly increase (Figure 2C). Spore generation from the three *T. asperellum* strains were the highest in nutrient broth with 1% of peptone, and started to drop in media with 2% of peptone (Figure 2D). Furthermore, cultivation at 28 ^{o}C for 15 days on rice husks as a carrier maximized spore generations of Tr.6, Tr.7, and Tr.8 strains (Figure 3E-H). Overall, cultivation of Tr.6, Tr.7, and Tr.8 strains with 2% of sucrose, 1% of peptone on rice husks, at 28^{o}C for 15 days was the best condition for producing conidia in this study.

Optimizing formulation ingredients

Although spore is the most resilent phage in the life cycle of microorganism, their viabilities can be decreased quickly in natural conditions, leading to shorten shelf life of microbiology-based products. The conidial survivals can be preserved by addition of stablizers such as Glycerol, carboxymethyl cellulose (CMC), and Tween 80. To define the optimal concentration of stablizer, different concentrations of Glycerol, CMC, and Tween 80 were added to broth containing Tr.6, Tr.7, and Tr.8 conidia with a ratio of 1:1:1. The conidial suspension were kept at room temperature (25-28^{o}C) and the percent of germinations were assessed at day 0, day 10, day 30, and day 60 by culturing an alequoes on PDA plates. As shown in Figure 3, conidial viability were negatively correlated with storage duration. The viable conidia were gradually reduced during the sampling period. Different concentrations of stablizers had significantly distinct effects on the conidial survial. In general, the higher the concentration of stabilizer was, the greater the numbers of viable spores were. The percentage of conidial germination were slightly decreased (~ 96%) after 10 days (Figure 3). After 30 days, the survival rates ranged from 60% - 90%, depending on stablizing conditions. After two months, the numbers of viable conidia slightly decreased in conditions shown in Figure 3A (66.6% vs. 63.3%) and in Figure 3C (83% vs. 80%), but considerably reduced in Figure 3B, indicating that conditions presented in Figure 3A & 3C were stable for preserving conidia. The conidial suspension supplemented with 10% glycerol, 0.2% CMC, and 0.3% Tween 80 (Figure 3C) were, therefore, selected for further study.

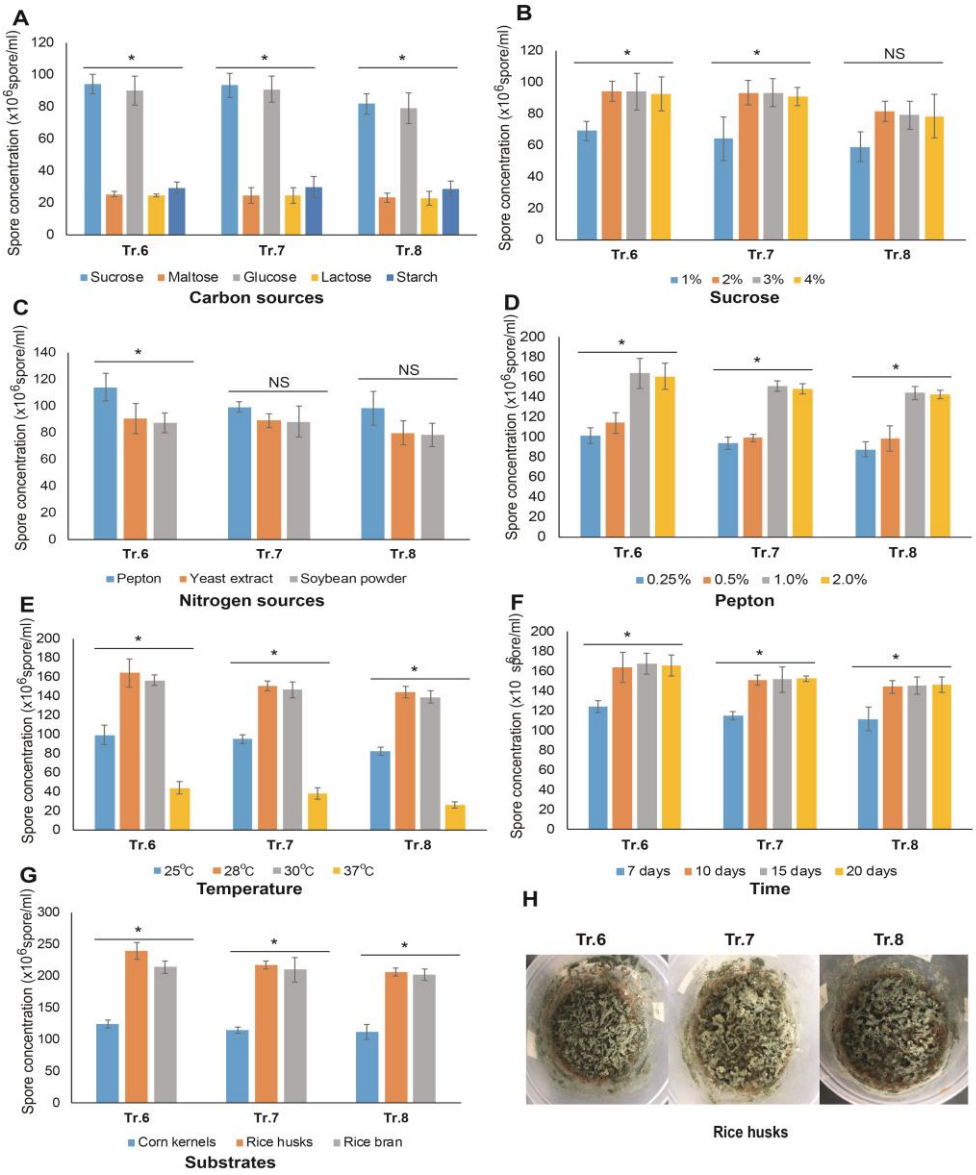

Figure 2. Optimal conditions for spore generations of *T. asperellum* Tr.6, Tr.7, and Tr.8 strains. (A&B) carbon sources and concentraion of the best source. (C&D) nitrogen sources and concentraion of optimal source. (E) Culture temperature. (F) culture duration. (G) culture substrates. (H) reprentive pictures of Tr.6, Tr.7, and Tr.8 growths on rice husks. One-way ANOVA was used to evaluate statistically significant. * = significant difference among tested conditions. NS = non-significant.

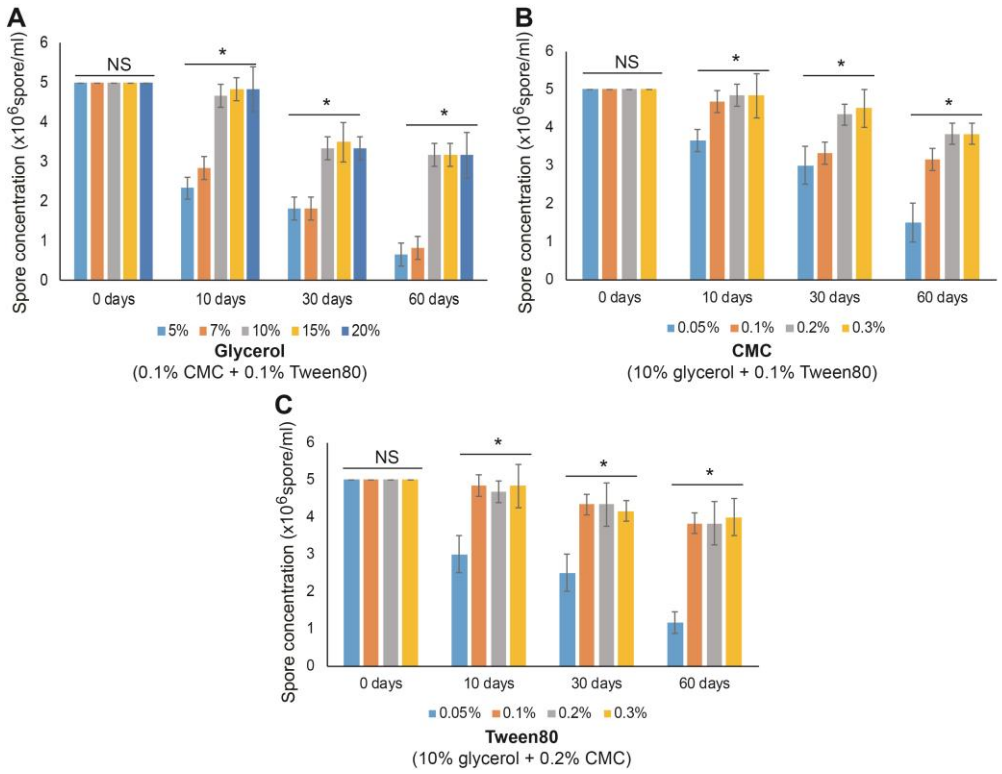

Figure 3. Optimal mixtures of stablizers for preserving Tr.6, Tr.7, and Tr.8 conidia. (A) Optimal concentration of glycerol. (B) Optimal amount of carboxymethyl cellulose (CMC). (C) Optimal concentration of Tween 80. One-way ANOVA was used to evaluate statistically significant. * = significant difference among tested conditions. NS = non-significant

Preventive effects of formulated conidia on citrus green mold and anthracnose

The preventive effects of the formulated conidia were assessed by pre-treated citrus fruits or leaves with water (negative control), or 1%, 0.5%, or 0.3% of conidial suspention in water. The pre-treated subjects were then infected with either *P. digitatium* or *C. gloeosporioides*, and observed for disease symptoms at day 3, 7, and 10. As presented in Fig. 4, orange fruits or leaves pre-treated with water showed disease symptoms at day 3 post infection with *P. digitatium* or *C. gloeosporioides*, whereas the ones soaked with different concentrations of the formulation before challenging with *P. digitatium* or *C. gloeosporioides* did not display any sign of infection. The disease symstoms, however, appeared on citrus fruits and leaves, which were treated with the lowest concentration of the conidial suspension (0.3%) at day

7. The green mold and anthracnose diseases were not observed on the orange fruits or leaves treated with 1% and 0.5% dilution of the conidial preparations at day ten post infection of *P. digitatium* and *C. gloeosporioides* (data not shown), suggesting that the formulation protected citrus from green mold and anthracnose development in a dose-dependent manner.

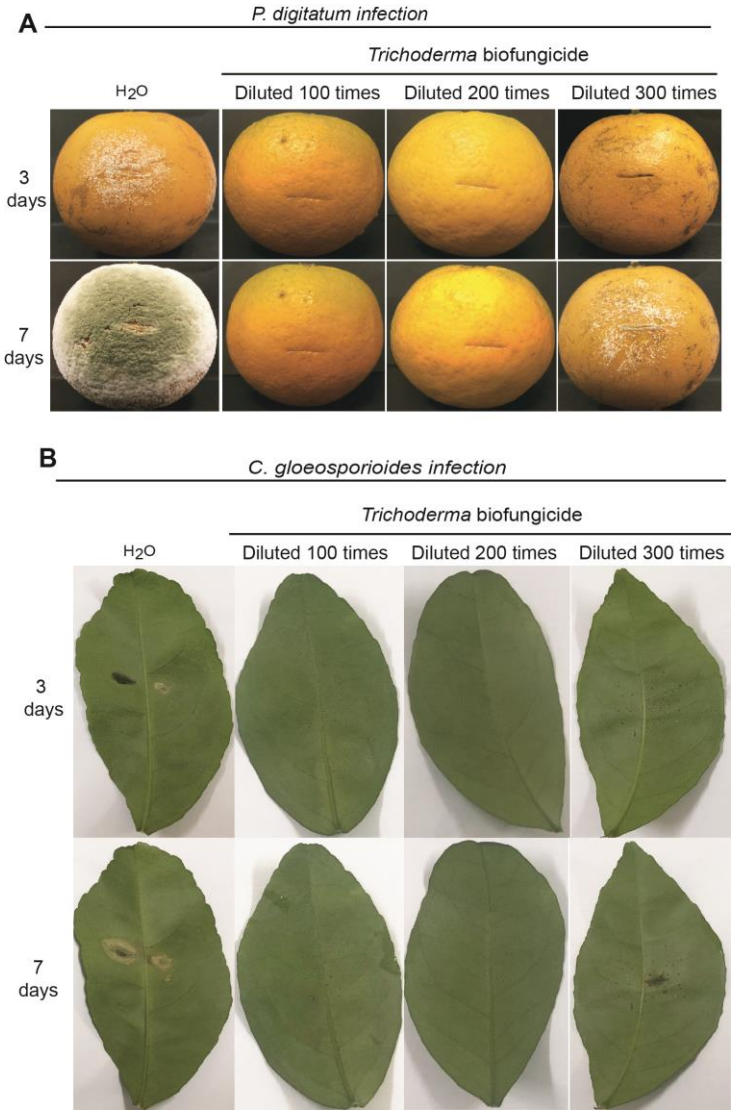

Figure 4. Formulated conidia of *T. asperellum* Tr.6, Tr.7, and Tr.8 strains prevented the development of citrus green mold and anthracnose. (A) Conidial formulation inhibited *P.digitatium* infection on orange fruits. (B) Conidial suspension protected citrus leaves from *C. gloeosporioides*

Discussion

Fungal pathogens are the major threads of citrus crop loss, which can result in economic loss of up to 50% of total production. Fungicides have been used widely to protect harvested fruits from spoilage. The use and misuse of fungicides, however, raised public concerns because of their risks for environment and human health. Therefore, biocontrol formulations, as an alternative of fungicides, have been actively investigating. Here, *T. asperellum* Tr.6, Tr.7, and Tr.8 strains simultaneously inhibited *P. digitatum* and *C. gloeosporioides* growths by 99% and 77%, respectively. Optimal conditions for formulated conidia and efficacy of the *T. asperellum*-based formulation in controlling citrus green mold and anthracnose were established. Altogether, these data indicated a potential application of the formulated conidia in preventing citrus crop loss caused by both preharvest and postharvest diseases.

C. gloeosporioides causes anthracnose on a wide range of hosts (470 genera), which is characterized by dark spots on leaves, stems, inflorescences, fruits, and developing shoots. *C. gloeosporioides* can spread quickly on the rainy season, and damage the whole orchard if not managed properly. *C. gloeosporioides* is well-characterized as the infectious agent of pre-harvest and post-harvest anthracnose in mango, papaya, guava, custard apple, and pomegranate (Sharma and Kulshrestha, 2015). Additionally, *C. gloeosporioides* has been identified as causing anthracnose on citrus fruits in Australia (Wang *et al*., 2021), in Tunisia (Rhaiem and Taylor, 2016), Portugal (Ramos *et al*., 2016), and Mexico (Cruz-Lagunas *et al*., 2020), and as the infectious agents of citrus post-bloom fruit drop in Brazil (Lima *et al*., 2011), respectively.

P. digitatum is the causal fungus of postharvest citrus fruit green mold, which can account for 90% of the total citrus postharvest losses. *P. digitatum* has a limited host range and mainly infects mature fruits of the *Rutaceae* family. *P. digitatum* genome sequences is the first genome sequencing of the phytopathogenic *Penicillium* species. The small gene content (26 Mb) in *P. digitatum* genome may, in part, explain the reason behind its limited host range. The high similarity of the whole genome sequences among globally distributed *P. digitatum* isolates indicate that geographical factors were trivial to *P. digitatum* development and that a single lineage in *P. digitatum* was expanded globally (Cheng *et al*., 2020).

The potential of *T. asperellum* species as biocontrol of *C. gloeosporioides* infection have been reported in several studies. Most of them demonstrated that *T. asperellum* strains inhibited *C. gloeosporioides* growth by 80% to 100% (Shang *et al*., 2020), (Ram ŕez-Olier *et al*., 2019), de los Santos-Villalobos *et al*., 2013), whereas one showed that *T. asperellum* T2-31 strain

could suppress *C. gloeosporioides* extension by only 23% (De la Cruz-Quiroz *et al*., 2018). The *T. asperellum* Tr.6, Tr.7, and Tr.8 strains in our collection displayed antagonistic activities of 73% to 80%, which is consistent with most of the studies describing the inhibitory effects of *T. asperellum* strains on *C. gloeosporioides* development. The anti-*P. digitatum* activities of *T. asperellum* strains other than those in our collection, however, have not been reported yet. The results of coculture assays in this study were comparable with our previous report that is the *T. asperellum* Tr.6, Tr.7, and Tr.8 strains repressed *P. digitatum* growth by 95% to 99%. Together, the *T. asperellum* Tr.6, Tr.7, and Tr.8 strains showed potential as biocontrol agents against infectious diseases caused by *C. gloeosporioides* and *P. digitatum.*

Glucose is a common source of carbons used in microbial fermentation. Glucose, therefore, has been compared with other carbon sources in *Trichoderma* cultivation. Rossi-Rodrigues *et al.* (2009) showed that growth rates of *T. hamatum, T. harzianum, T. viride*, and *T. longibrachiatum* cultured with glucose were 12% faster than when cultivated in the presence of sucrose (Rossi-Rodrigues *et al*., 2009). Our results, however, showed that *T. asperellum* Tr.6, Tr.7, and Tr.8 strains grew slightly faster in sucrose than they did in glucose, although the difference was not statically significant. Since Rossi-Rodrigues *et al.* (2009) did not evaluated their results by statistic tests, hence, the difference between glucose and sucrose might be not significant. Haque *et al.* (2020) demonstrated that their *T. asperellum* strain expanded fastest at the temperatures between 25 and 30^{o}C, which was consistent with the optimal temperature in this study.

Most *Trichoderma* preparations are commercially formulated as wettable powders (55.3%) composing of a given concentration of dried fungal conidia spores in a fine dust. Granular (13.6%), liquid (10.3%), and solid (6.2%) formulations are the second most common form of *Trichoderma*-based products. Emulsions, concentrated liquid suspensions, dry flowable pellets, powder or talc are other formations of *Trichoderma* formulations. The wettable powders, granules, emulsions, suspensions of *Trichoderma* can be dissolved in water for applications such as spray (ground and aerial), root drench, dip, seed treatment, irrigation, hydroponics; whereas the pellets, dry flowables and solid formulations can be directly applied to the soil at time of seeding or transplant.

Tween is a surfactant, which is widely used in spore suspension to promote spore viability and solution (Ran *et al*., 2016). Glycerol is a humectant, which usually used in laboratories to preserve microorganisms for long term (Nakasone *et al*., 2004). In this study, a liquid suspension for foliar spray application were prepared by supplementing the *Trichoderma* broth with an optimal mixture of stabilizers including Tween80 and glycerol, leading to

stabilizing conidial viability for up to 80%. This was the simple, cheap, and effective method for preparing *Trichodema* formulation.

We were the first group to demonstrate that strains of *T. asperellum* species effectively inhibited *P. digitatum* growths, and that *T. asperellum* strains protected citrus crops from infected by *C. gloeosporioides* and *P. digitatum*, which are the main causes of citrus preharvest and postharvest diseases, respectively. A simple, cheap, and effective method for liquid formulation of *T. asperellum* was also the first time described in this study. Field trials to evaluate the efficacy of this formulation was ongoing.

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