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Trichoderma asperellum (NST-009): A Possible Thai Native Antagonistic Fungus for Managing White Root Disease of Rubber Trees (*Hevea brasiliensis*)

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ABSTRACT

Background: *Rigidoporus microporus* causes white root disease, which is one of the most harmful diseases in rubber trees in Thailand. The objectives of this study were to determine the efficacy of *T. asperellum* NST-009 and its antifungal metabolite in inhibiting *R. microporus* mycelial development and efficacy of *T. asperellum* NST-009 in controlling white root disease of rubber trees in an open-field house experiment.

Methods: Four native strains of *T. asperellum* from Nakhon Si Thammarat Province and a commercial strain of Thailand were used in this study. This study was conducted at Agricultural Microbial Production and Service Center, Walailak University, Nakhon Si Thammarat, Thailand, during the period 2017-2020.

Result: *T. asperellum* NST-009 significantly inhibited *R. microporus* mycelial growth by 77.07% *in vitro* and its antifungal metabolite from the culture filtrate of *T. asperellum* NST-009 inhibited mycelial growth by 92.31%. *T. asperellum* NST-009 reduced the disease severity index by 76.38% in the open-field house experiment compared to the inoculated control. Furthermore, *T. asperellum* NST-009 was found to survive in rhizosphere soil at 4.50×10^5 CFU/g soil and colonized the roots at 100.00%.

Key words: Biocontrol, Parasitism, Rubber tree, *Trichoderma* strains, White root disease.

INTRODUCTION

The rubber tree (*Hevea brasiliensis*) is one of Thailand's most produced agricultural plants because it provides important material for a variety of applications including the automobile industry. The latex sap from the rubber tree is used for the production of insulating handles, tires, balls, balloons, and shock absorbers. More than 92% of the world's natural rubber is produced on plantations in Southeast Asia, including Thailand, Indonesia, and Malaysia (Diaby *et al*. 2011; Rukkhun *et al*. 2021). However, the white root disease, the most destructive root disease in rubber tree, caused by *Rigidoporus microporus* (Fr.) Overeem is a major problem in rubber plantations causing significant economic damage. It is the most destructive root disease in rubber plantations in Sri Lanka, India, Indonesia, Malaysia, Thailand, and West and Central Africa (Jayasuriya and Thennakoon, 2007). In the absence of woody substrates, *R. microporus* forms several white, flattened mycelial strands that expand and spread rapidly through the soil (Kaewchai and Soytong, 2010). Since the rhizomorph and infection sites are below ground level, early stages of infection through contact with a disease source, such as contaminated roots, dead stumps, or wood debris of root pathogens, are difficult to detect. Trees that die frequently go unnoticed for a long time, with no noticeable aerial (above-ground) signs (Ogbebor *et al*. 2013). This will ultimately lead to the death of many trees, as well as the destruction of the entire stand. The disease persists for a long time in dead or alive root debris, causing new infections in healthy plants (Kaewchai and Soytong, 2010). Cultural methods and chemical fungicides such as tridemorph, cyproconazole, hexaconazole, propiconazole, and fenicolonil are commonly used to treat this disease. These chemical fungicides, on the other hand, are known to affect human health, pollute the atmosphere and leave residues in agricultural soils and environments. Chemical fungicide resistance has also been observed for several plant pathogenic fungi. Furthermore, chemical fungicides are expensive. As a result, biological regulation is a viable option for reducing costs, environmental issues and health risks.

Trichoderma strains have been shown to be effective biological control agents against *Alternaria*, *Botrytis*, *Bipolaris*, *Cercospora*, *Colletotrichum*, *Corynespora*, *Curvularia*, *Phomopsis*, *Pythium*, *Phytophthora*, *Ganoderma*, *Rhizoctonia*, *Fusarium*, *Sclerotium*, *Sclerotinia* and *Rosellinia*. (Abo-Elyousr *et al*. 2014; Athul and Jisha, 2014; Fitrianingsih *et al*. 2019; Izzati and Abdullah, 2008; Nawrocka

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et al. 2018; Redda *et al*. 2018; Wonglom *et al*. 2019). *T. asperellum* NST-009 is a native fungus isolated from forest soil in Nakhon Si Thammarat, Thailand. This strain has been shown to be effective in controlling *Phytophthora* leaf fall in a rubber tree (Promwee *et al*. 2017). It is currently being recommended to farmers for plant disease control and growth promotion in a variety of plants, including rice, durian, oil palm and vegetables, by the Agricultural Microbial Production and Service Center at Walailak University and is being distributed to farmers throughout Thai provinces. There have been no records of native *T. asperellum* NST-009 used to manage rubber-tree white root disease. Hence, the objectives of this study were: 1) to determine the efficacy of *T. asperellum* NST-009 and its antifungal metabolite in inhibiting *R. microporus* mycelial development and 2) to determine the efficacy of *T. asperellum* NST-009 in controlling white root disease of rubber trees in an open-field house experiment.

MATERIALS AND METHODS

Trichoderma strains and the *Rigidoporus* pathogen

Four native strains of *T. asperellum* (known as *T. harzianum*) from Nakhon Si Thammarat (*T. asperellum* NST-003, NST-009, NST-028 and NST-353) and the commercial strain of Thailand (*T. asperellum* CB-Pin-01) were used in this study (Promwee *et al*. 2017; Charoenrak *et al*. 2019; Unartngam *et al*. 2020). The pathogen (*R. microporus* Tha-01) was collected from the Walailak University's Agricultural Microbial Production and Service Center. The *Trichoderma* strains and the *Rigidoporus* pathogen were subcultured on potato dextrose agar (PDA) for 5 days at room temperature (28±2°C). Koch's postulates were used to confirm the symptoms of white root disease caused by *R. microporus*, and the morphological and reproductive characteristics of *R. microporus* were examined using a light compound microscope and a scanning electron microscope (SEM) (Kaewchai *et al*. 2010). This study was conducted at Agricultural Microbial Production and Service Center, Walailak University, Nakhon Si Thammarat, Thailand, during the period 2017-2020.

Dual culture test

All strains of *T. asperellum* were tested for their ability to inhibit the mycelial growth of *R. microporus*. A sterile cork borer (3 mm diameter) was used to cut a five-day-old *T. asperellum* on PDA and the agar plug of *T. asperellum* was mounted on one side of the PDA Petri dish (9 cm diameter). Then, on opposite sides of the PDA Petri dish, a 3-mm-diameter plug of 5-day-old *R. microporus* was mounted, and the plates were incubated for 5 days at 28±2°C. Using a completely randomized design (CRD) of four replications, the experiment was performed and replicated twice.

The per cent inhibition of mycelial growth was determined using formula (1):

$$\text{Inhibition (\%)} = \frac{\text{RC-RT}}{\text{RC}} \times 100 \quad \text{.....(1)}$$

Where,

RC: Represents *R. microporus* radial growth in the untreated regulation.

RT: Represents *R. microporus* radial growth during the procedure.

Scanning electron microscopy analysis

Using a SEM (JEOL, JSM5600LV, England), the high-efficiency strain of *T. asperellum*, which was able to inhibit the mycelia of *R. microporus* in the dual culture test, was studied for its ability to parasitize the mycelia of *R. microporus*. *T. asperellum* and *R. microporus* were grown on PDA in a dual culture test. After a colony of *R. microporus* was targeted by *T. asperellum* mycelia, the activity zone samples were cut into small pieces (0.5×0.5 cm), fixed in 2.5% glutaraldehyde for 24 h at 4°C, rinsed with distilled water, and dehydrated in a 30-100% alcohol sequence. The samples were dried in a critical point dryer before being coated with gold using a sputter coater (Nanotech, Sempreps, England). SEM was used to analyse the coated samples right away.

Antifungal metabolite determination

Twenty-five mycelial agar plugs (7 mm diameter) obtained from the margins of developing colonies of *T. asperellum* grown on PDA were inoculated into a 3 L Erlenmeyer flask containing 1 L of 1/5 strength potato dextrose broth (PDB). The flask was then incubated for 28 d at 28±2°C. The spores and mycelia of *T. asperellum* were then separated from the broth culture using 0.45 m Whatman No.1 filtration. Ethyl acetate was used to extract the culture filtrates before evaporation at 40°C using a rotary vacuum evaporator (EYELA, Japan). The agar dilution method was used to assess antifungal metabolites (crude extracted substances) for their ability to inhibit the mycelial growth of *R. microporus* on PDA. Each antifungal metabolite was dissolved in 2% DMSO, combined with PDA to a final concentration of 500 mg/l and poured into a Petri dish. The *R. microporus* mycelial agar disc was then placed in the center of a solidified agar plate and incubated for 5 days at 28±2°C. The experiment was repeated twice with four replications using a CRD. *R. microporus* colony diameter was measured and the inhibition percentage of mycelial growth was calculated using formula (2):

$$\text{Inhibition (\%)} = \frac{\text{DC-DT}}{\text{DC}} \times 100 \quad \text{.....(2)}$$

Where

DC: Represents the mean mycelial diameter of *R. microporus* in the control treatment.

DT: Represents the mean mycelial diameter of *R. microporus* in the tested treatment.

Disease control under open-field house experiment

Preparation of the *R. microporus inoculum*

R. microporus was subcultured on PDA for five days at 28±2°C and *R. microporus* inoculums were prepared using the modified technique described by Kaewchai and Soytong (2010). Ten *R. microporus* mycelial plugs were cultured in sterilized inoculum medium (100 g sawdust, 3 g rice bran, 2 g

glucose and 100 mL distilled water) in each plastic bag for 30 days at 28±2°C.

T. asperellum fresh culture preparation

T. asperellum strains were subcultured on PDA for five days at 28±2°C. A simple technique was used to prepare fresh *T. asperellum* cultures. An electric rice cooker was used to cook rice and water (3:2 by volume). The hot cooked rice was placed in clear plastic bags (250 g/bag) and allowed to cool slightly above room temperature. In a plastic bag, ten mycelial plugs (0.8 cm diameter) of the *T. asperellum* colony growing on a PDA dish were inoculated and mixed with the cooked rice. Each bag's open end was secured with a rubber band and a needle was used to puncture the attached area (25 holes/bag). Before use, all bags were incubated at 28±2°C for seven days under fluorescent light (12 h/day) (Charoenrak *et al*. 2019).

Disease severity assays

Fresh *T. asperellum* culture was added to the planting medium (10 kg of soil from a rubber plantation in Nakhon Si Thammarat mixed with 2 kg of cow manure) at 100 g per pot and incubated for seven days. The 6-month-old rubber tree cultivar RRIM 600 was then planted in a 15-inch-diameter plastic container and a bag of *R. microporus* inoculums was placed in a planting pot next to root system of the rubber tree. In this analysis, CRD with four replications per treatment and three plants per replication was compared to fungicide (cyproconazole 10% w/v), control 1 (with only pathogen) and control 2 (without pathogen). Six months after inoculation with *R. microporus*, the disease was observed on the rubber tree. The experiment was repeated twice.

The severity of the disease was divided into six levels (0-5): level 0 = stable, green leaves; level 1=1-25% yellow leaves; level 2=26-50% yellow leaves; level 3=51-75% yellow leaves; level 4=76-100% yellow leaves and level 5= dead tree (Kaewchai and Soytong, 2010). The disease severity index (DSI) was calculated using the formula (3):

$$DSI\ (\%) = \frac{\sum(\text{Level} \times \text{Amount of trees})}{\text{Maximum level} \times \text{Total trees}} \times 100 \quad .....(3)$$

Rubber root infected and covered by mycelia of *R. microporus*

Root infection and coverage by *R. microporus* mycelia were estimated six months after the plants were inoculated with *R. microporus* inocula and classified into five levels (0-4) as follows: level 0= no infection and colonization, level 1=1-25% of infection and colonization, level 2=26-50% of infection and colonization, level 3=51-75% of infection and colonization and level 4=76-100% of infection and colonization. The *R. microporus* root infected and covered by the mycelia index (RICI) was calculated using formula (4):

$$RICI\ (\%) = \frac{\sum(\text{Level} \times \text{Amount of roots})}{\text{Maximum level} \times \text{Total roots}} \times 100 \quad .....(4)$$

Root colonization of *T. asperellum*

Root colonization of *T. asperellum* was studied using Martin's medium six months after inoculation with *R. microporus*. The rubber root was cut into ten pieces and soaked in 0.525 per cent sodium hypochlorite (Clorox®) for 5 min. The rubber root was then washed three times with sterilized distilled water. Five rubber root pieces were dried with sterilized paper and placed in a Petri dish on Martin's medium. The dishes were sealed with paraffin film and incubated for four days at 28±2°C. The percentage of root colonization was then calculated. This experiment used a CRD with four replications and five rubber root per replication.

Population of *T. asperellum* in rhizosphere soil

The population of *T. asperellum* in rhizosphere soil was studied at six months after inoculation with *R. microporus* using the dilution plate technique and Martin's medium**.** In a 250 mL flask containing 90 mL of sterile water, ten g of each rhizosphere soil was added and mixed for 30 min with shaking at 120 rpm. The soil suspension was then diluted 10^{-2}-10^{-4} times before being placed on the surface of Martin's medium in a Petri dish with 0.1 mL of the dilution. Before the plates were coated with paraffin film and incubated at 28±2°C for 4 days, the soil suspension was spread on the surface of the medium with a sterile glass rod. The growth of *T. asperellum* on agar was then estimated. This experiment used a CRD with four replications.

Statistical analysis

An analysis of variance (ANOVA) was performed on the results, followed by a comparison using Duncan's multiple range test ($P<0.05$).

RESULTS AND DISCUSSION

Effect of *T. asperellum* on growth of *R. microporus*

In a dual culture experiment, all strains of *T. asperellum* effectively inhibited *R. microporus* mycelia on PDA (70.82-77.07%), with *T. asperellum* NST-009 providing the highest percentage of mycelial growth inhibition (Table 1, Fig 1).

Table 1: Effect of *T. asperellum* on mycelial growth inhibition of *Rigidoporus microporus* after incubation at room temperature for 5 days.

Treatments	*R. microporus* inhibition (%)
T. asperellum NST-003	70.82^{c*}
T. asperellum NST-009	72.74^{b}
T. asperellum NST-028	77.07^{a}
T. asperellum NST-353	76.12^{a}
T. asperellum CB-Pin-01	74.67^{ab}
R. microporus	-

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

Parasitism of *T. asperellum* against *R. microporus*

Under SEM, the ability of *T. asperellum* to induce mycoparasitism on the mycelia of *R. microporus* revealed that the selected strain of *T. asperellum* NST-009 was able to parasitize the mycelia of *R. microporus via Rigidoporus* hypha colonization, *Rigidoporus* hypha drilling holes and conidia reproduction on *Rigidoporus* (Fig 2).

Effect of crude metabolites on *R. microporus* growth

The antifungal metabolites of all *T. asperellum* strains at 500 µg ml^{-1} effectively inhibited *R. microporus* mycelial growth (71.15-92.31%), especially antifungal metabolites of *T. asperellum* strain NST-009, which showed the highest percentage of mycelial growth inhibition (Table 2 and Fig 3).

***T. asperellum* controls white root rot disease in open field conditions**

Disease severity

The effectiveness of *T. asperellum* in controlling white root disease in rubber trees was investigated under open-field conditions. The results showed that 180 days after the rubber

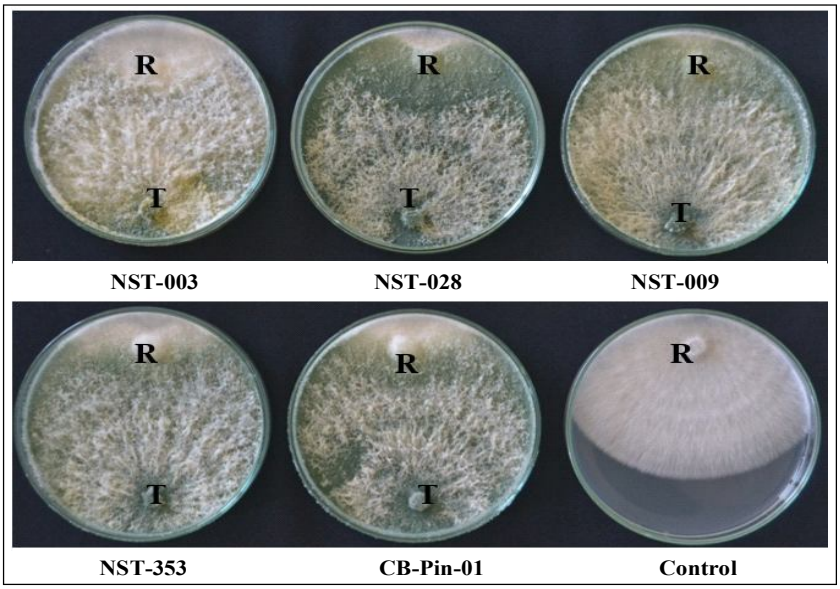

Fig 1: The efficacy of *Trichoderma asperellum* to inhibit and overgrow on mycelia of *Rigidoporus microporus* by a dual culture test at 28±2°C for 5 days; (T= *T. asperellum*; R= *R. microporus*).

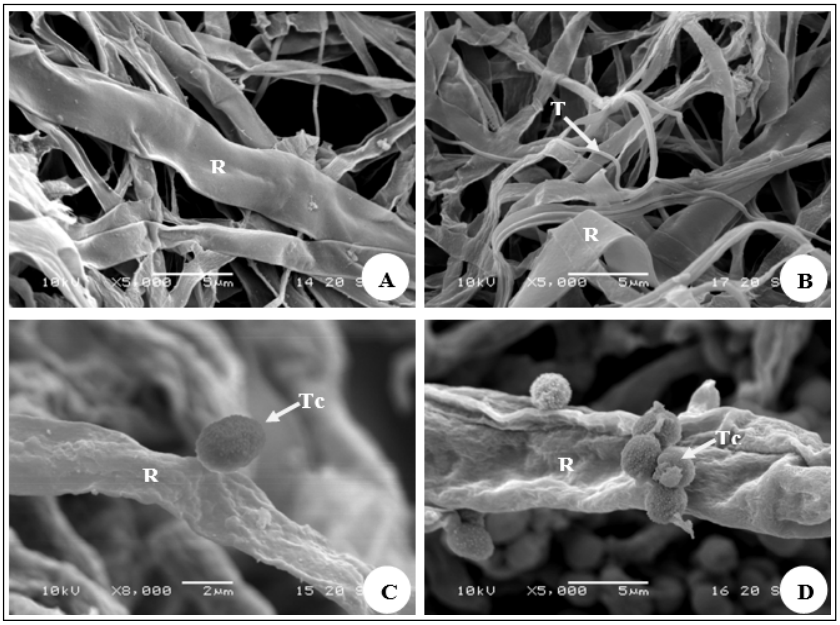

Fig 2: Scanning electron micrographs of *Trichoderma asperellum* strain NST-009 hyphae (T) interact with mycelium of *Rigidoporus microporus* (R) from the dual culture test: (A) Normal hypha of *R. microporus* cultured on PDA without *T. asperellum*, (B, C and D) Hypha of *R. microporus* were attacked by *T. asperellum* strain NST-009; Tc= conidia of *T. asperellum.*

trees were inoculated with *R. microporus* inoculum, all *T. asperellum* strains had a high efficacy in controlling white root disease with a low disease severity index (0.00–19.44%), especially the *T. asperellum* strains NST-028, NST-009 and CB-Pin-01 (commercial strain), which had a disease severity index similar to that of the fungicide (cyproconazol). The control 1 treatment, which was only inoculated with *R. microporus*, had the highest disease severity index (76.38%) (Table 3).

Rubber root infected and covered by mycelia of *Rigidoporus microporus*

Rubber roots infected and covered with mycelia of *R. microporus* were studied 180 days after inoculation with the inoculum of *R. microporus*. The results showed that treatment with *T. asperellum* caused significant root infection and was covered by mycelia of *R. microporus* index (RICI) as compared with the control 1 (with only pathogen). In particular, the treatments with *T. asperellum* strain NST-009, NST-028 and CB-Pin-01 provided the lowest RICI (0.00%), while control 1 had the highest RICI (64.58%) (Table 3).

Table 2: Effect of the crude extract of *Trichoderma* cultures on mycelial growth inhibition of *R. microporus* after incubation at room temperature for 5 days.

Treatments	*R. microporus* inhibition (%)
T. asperellum NST-003	71.15^{d}*
T. asperellum NST-009	84.62^{b}
T. asperellum NST-028	92.31^{a}
T. asperellum NST-353	76.92^{c}
T. asperellum CB-Pin-01	82.69^{b}
R. microporus	-

*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).

Trichoderma root colonization

Root colonization of *T. asperellum* was determined after application of *T. asperellum* for 180 days. Treatment with *T. asperellum* strains NST-028 and NST-009 resulted in the highest rate of root colonization (100.00%), followed by NST-353 (95.00%), CB-Pin-01 (85.00%) and NST-003 (75.00%), which showed significant differences compared to control 1, control 2 and cyproconazole treatments, which did not find *Trichoderma* strains colonize the roots (Table 3).

Trichoderma population

The population of *Trichoderma* strains in the planting medium was studied using the dilution plate technique with Martin's medium. The results showed that the treatments with *Trichoderma* strain provided the population at 1.25×10^{6}-3.25×10^{6}, 4.00×10^{5}-12.75×10^{5} and 2.00-4.50×10^{5} CFU/g of the planting medium at 60, 120 and 180 days, respectively, after inoculation with a fresh culture of *T. asperellum*. On the other hand, treatments with fungicide (cyproconazole), control 1 (with the pathogen) and control 2 (without pathogen) were not found in the population of *Trichoderma* strains in the planting medium (Table 4).

In this study, *T. asperellum* NST-009 displayed strong antifungal activity against *R. microporus* in a dual culture assay. The dual culture assay revealed competition and mycoparasitism of *T. asperellum*. Crude metabolites of *T. asperellum* NST-009 showed fungicidal activity against *R. microporus*, revealing antibiosis. Application of fresh *T. asperellum* NST-009 reduced the disease severity index

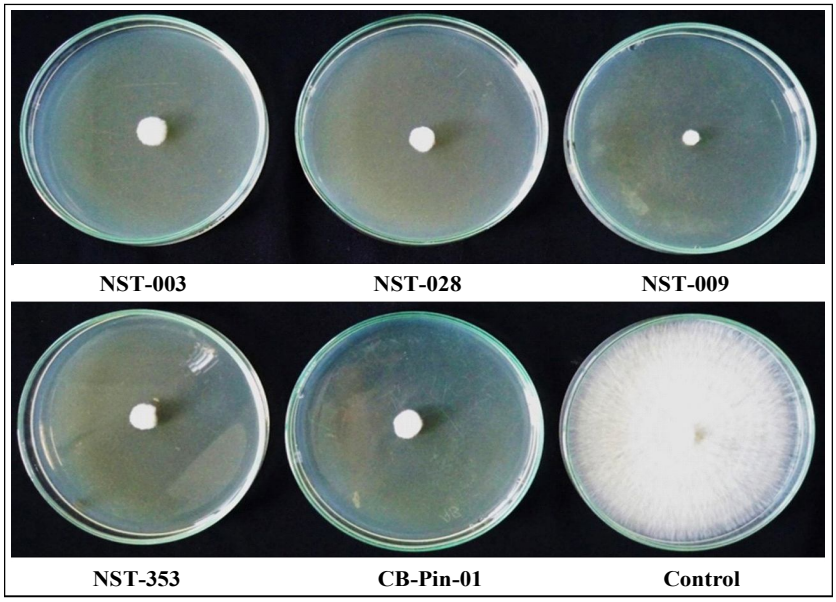

Fig 3: The efficacy of antifungal metabolites of *Trichoderma asperellum* at concentration of 500 mg/L to inhibit the mycelia growth of *Rigidoporus microporus* after incubation at 28±2°C for 5 days.

Table 3: Disease severity index of white root disease (DSI), root infected and covered by mycelia of *Rigidoporus microporus* index (RICI) and *Trichoderma* root colonization (TRC) after the rubber tree inoculated with *R. microporus* under open-field house conditions for 180 days.

Treatments	DSI (%)	RICI (%)	TRC (%)
T. asperellum NST-003 + *R. microporus*	19.44^{b*}	10.42^{b}	75.00^{b}
T. asperellum NST-028 + *R. microporus*	0.00^{c}	0.00^{c}	100.00^{a}
T. asperellum NST-009 + *R. microporus*	0.00^{c}	0.00^{c}	100.00^{a}
T. asperellum NST-353 + *R. microporus*	11.11^{bc}	4.16^{c}	95.00^{a}
T. asperellum CB-Pin-01 + *R. microporus*	0.00^{c}	0.00^{c}	85.00^{ab}
Cyproconazol + *R. microporus*	0.00^{c}	0.00^{c}	0.00^{c}
Control 1 (+ *R. microporus*)	76.38^{a}	64.58^{a}	0.00^{c}
Control 2 (- *R. microporus*)	0.00^{c}	0.00^{c}	0.00^{c}

*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).

Table 4: *Trichoderma* population in the planting medium at 60, 120 and 180 days after inoculation with fresh culture of *T. asperellum* in testing to control white root disease of rubber tree under open-field house conditions.

Treatments	*Trichoderma* population (CFU/g planting medium)		
	60 days	120 days	180 days
T. asperellum NST-003 + *R. microporus*	$2.25\times10^{6a*}$	4.00×10^{5b}	2.00×10^{5b}
T. asperellum NST-028 + *R. microporus*	2.75×10^{6a}	6.75×10^{5b}	3.50×10^{5ab}
T. asperellum NST-009 + *R. microporus*	3.25×10^{6a}	12.75×10^{5a}	4.50×10^{5a}
T. asperellum NST-353 + *R. microporus*	1.25×10^{6a}	4.75×10^{5b}	3.25×10^{5ab}
T. asperellum CB-Pin-01 + *R. microporus*	2.00×10^{6a}	5.00×10^{5b}	3.00×10^{5ab}
Cyproconazole + *R. microporus*	0.00^{b}	0.00^{c}	0.00^{c}
Control 1 (+ *R. microporus*)	0.00^{b}	0.00^{c}	0.00^{c}
Control 2 (- *R. microporus*)	0.00^{b}	0.00^{c}	0.00^{c}

*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).

caused by *R. microporus* under open-field conditions and colonization of the rubber tree rhizosphere.

Trichoderma species are known as potent biocontrol agents against several plant diseases because of their capacity to compete for nutrients and space and emit volatile antifungal compounds against plant pathogens (Gangwar and Singh, 2018; Baiyee *et al*. 2019b; Wonglom *et al*. 2020). Based on the results of this study, *T. asperellum* NST-009 competed for nutrients and space when co-cultured with *R. microporus*. These results support the findings of Jayasuriya and Thennakoon (2007) and Kaewchai and Soytong (2010) that *Trichoderma* strains had a competitive ability to inhibit mycelial growth of *R. microporus*. Moreover, *Trichoderma* strains can inhibit the mycelial growth of several plant pathogens, such as *Alternaria alternata, Botrytis cinerea*, *Corynespora cassiicola, Curvularia oryzae, Fusarium solani*, *F. oxysporum*, *Pythium aphanidermatum*, *Rhizoctonia solani*, *Rosellinia necatrix* and *Sclerotium rolfsii* (Intana *et al*. 2003; Rosa and Herrera, 2009; Nallathambi *et al*. 2009; Sunpapao *et al*. 2018; Baiyee *et al*. 2019b, Wonglom *et al*. 2019; Wonglom *et al*. 2020). This dual culture test suggested that the two mechanisms of *T. asperellum* were competition for nutrients and space, as well as mycoparasitism. *Trichoderma* spp. can produce antifungal metabolites that restrict fungal growth. The results of this study demonstrated that crude metabolites of *T. asperellum* NST-009 inhibited the fungal growth of *R. microporus*.

CONCLUSION

The *Trichoderma asperellum* strain NST-009 is native to southern Thailand. *T. asperellum* NST-009 effectively inhibited *R. microporus* mycelial growth more than the Thai commercial strain *T. asperellum* CB-Pin-01 in both dual culture and crude extract tests. *T. asperellum* NST-009 exhibited mycoparasitism under a scanning electron microscope. *T. asperellum* NST-009 exhibited disease severity indexes comparable to those of cyproconazole fungicide in an open-field house experiment. Furthermore, *T. asperellum* NST-009 showed root colonization that was protective against *R. microporus* infection of rubber trees and could survive in soil.

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