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Characterization of double-stranded RNA in *Trichoderma* spp. isolates in Chiang Mai province

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One hundred and fifty-six isolates of *Trichoderma* spp. were collected from mushroom growth media, farm lands and commercial biological product using dilution plate method were examined for the presence of dsRNA. Two of these isolates contained double-stranded RNA (dsRNA) elements were detected. One dsRNA segment of 1000 bp and two of dsRNA segments of 700 and 1100 bp were detected from isolate TM10 and TM20 respectively. To eliminate dsRNA these isolates namely TM10h and TM20h were subjected to heat therapy by alternately incubated at 37 °C for 3 hours and room temperature (28-30 °C) interval for 24 hours and then subcultured for 10 times. It was found that both fungal isolates were improved their growth rate and sporulation, dsRNA was still appeared. Production of exochitinase from *Trichoderma* spp. isolates: T15, T42, T75 (free from dsRNA); TM10, TM20 (contained dsRNA) and TM10h, TM20h (contained dsRNA after heat treatment) were evaluated. There was not significant of their enzyme production among these isolates. By other hand all 7 isolates were evaluated for antagonistic activity to *Sclerotium rolfsii* by dual culture technique. Percentage of growth inhibition of free dsRNA isolates, T15, T42 and T75 were 71.67, 54.93 and 60.79% which higher than dsRNA-containing isolates TM10, TM20, TM10h and TM20h that inhibition percentages were 25.42, 24.62, 28.52 and 26.74% respectively. The results of greenhouse experiment indicated that *Trichoderma* spp. T15 and T75 showed a high ability of inhibitory effects on plant pathogen that percentages of disease incidences were 25.00% for both of the isolates and 48.75% for the T42 isolate while TM10, TM20, TM10h and TM20h isolates had lower ability to inhibit pathogen that the disease incidence were 72.50, 66.25, 62.25 and 63.75%, respectively. It was obvioused the appearance of dsRNA in both *Trichoderma* isolates, TM10 and TM20 resulted in the reduction of their growth rate, sporulation and biological control efficacy.

Key words: *Trichoderma*, dsRNA, *Sclerotium rolfsii*, biocontrol, dual culture

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Introduction

Double-stranded RNA (dsRNA) are widespread in all classes of plant pathogenic fungi (Buck, 1986). However, in most cases these infections have not been associated with apparent disease symptoms or other phenotypes. In some phytopathogenic fungi, however, dsRNA viruses are associated with reduced virulence (hypovirulence) and other phenotypes such as reduced growth, sporulation, or pigmentation (Nuss and Koltin, 1990).

Trichoderma species are common fungi found in many cultivated and natural soils, and have long been known for their capacity to reduce plant diseases caused by soil-borne fungi (Baker and Cook, 1974; Whipps and Lumsden, 2001) and some have been tested for biological control potential in many field and greenhouse trials as well as commercial formulation available worldwild. Interestingly in present day extensive studies on the biological roles of dsRNA have been conducted. In *Cryphonectria paracitica*, the chestnut blight fungus. It was found that the conversion of a virulent strain to a hypovirulent one coincided with transmission of dsRNA by hyphal anastomosis (Anagnostakis and Day, 1979). Because of unconsistency of many reports concerned the influent of dsRNA contained with many fungi including *Trichoderma* spp. which recognized as biological control agent of plant pathogens worldwide. This study reported the presence and effect of dsRNA molecules in *Trichoderma* isolates derived from mushroom growth media and farm lands in Chiang Mai Province and commercial biological product. Additionally, some characteristic of dsRNA contained isolate including their morphology and biological control activity both in laboratory and greenhouse were compared with free dsRNA isolates and the issue concern their hypovirulent related to dsRNA was discussed.

Materials and methods

Fungal isolates

One hundred and fifty-six isolates of *Trichoderma* spp. were isolated from mushroom growth media, farm land and commercial biological product collected from 24 districts in Chiang Mai province using the dilution method.

Extraction and purification of dsRNA

The dsRNA was extracted from mycelial using the modified method of Jordan and Dodds (1985) and Valverde *et al.* (1990), grind 1.5 g of each sample in 2.0 ml of extraction buffer (2XSTE buffer 2% SDS and 1% PVP) with using -80 ^{o}C freezed mortar and added 400 µl of solution then transferred

to a 1.5 ml centrifuge tube and 400 µl phenol and mixture of chlroform:pentanol (24:1), then centrifuged at 4000 rpm for 30 min. Supernatant was then transferred to new tube and adjusted the concentration to equal of 16 % ethanol. For collection of dsRNA the solution was subjected to cycle of cellulose CF-11 chromatography and collected dsRNA were eletrophoresed on 1.5 % agarose gel and detected on a UV transilluminator.

Samples of this material were treated with various nucleases to identify their nature. These treatments were ribonuclease in 0.1X SSC (1XSSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), or in 2XSSC or with deoxyribonuclease in 40 mM Tris- HCl pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$ and 10 mM $CaCl_2$. The nucleic acids were incubated with the nucleases at 37 ^oC for 20 min.

Elimination of dsRNA

Mycelial plugs of dsRNA contained isolates were transferred to potato dextrose agar (PDA). The isolate were incubated at 37 ^oC for heat therapy to eliminate dsRNA (3 hours) then incubated in the room temperature for 24 hours, alternately and subcultured for 10 times. Each of heat treatment the treated isolates were evaluated for their mycelial growth, sporulation and the presence of dsRNA.

Enzymatic assay

Each *Trichoderma* isolate was cultured 50 ml liquid SM medium in 250 ml flask by inoculated with 1 ml of a conidial suspension (1×10^{10} conidia ml^{-1}) and incubated at 28 ^oC for 4 days. Chitinase activity was evaluated by incubated culture supernatants with 2.0 mM p-Nitrophenyl-N-acetyl-β-D-glucosaminine at 37 ^oC for 5 min. The released p-nitrophenol was measured using spectrophotometric method at 405 nm (Zaldivar *et al.*, 2001; Sigma Technical Service Department, 1996).

In-vitro and in-vivo test for antagonistic activity

To evaluation of antagonistic activity free dsRNA isolate and dsRNA contained isolate as well as heat treated dsRNA contained isolate for control *Sclerotium rolfsii* were used dual culture technique. The experiment was Completely Randomize Design (CRD) with 3 replications. Five millimeter discs of each isolate were removed from the edge of colonies in 5 days PDA cultures and placed on one side of a 90-mm-petri dishes containing PDA medium. Similar discs of *S. rolfsii* growth in the same manner were placed on the opposite

side of Petri-dishes for each isolate. Cultures were observed daily and the degree of antagonism was calculated according to Kucuk and Kivanc (2003).

In-vivo study, using potting soil bioassay. The soil was sterilized. The free dsRNA isolate dsRNA contained isolates or dsRNA associated isolate after heat treated was added in to soil infested with inoculums of the pathogen simultaneously at the ratio of 40:40 g (*Trichoderma* growth on sterilized sorghum seed: *S. rolfsii* growth on sterilized sorghum seed) in 800 g soil per pot, then surface sterilized soybean seeds with 3% sodium hypochlorite solution for 3 min, and 5 seeds were sown in each pot. Pre and post emergence of damping off were recorded as 0-4 which indicated symptomless, mild symptom, severe but no damping-off, dead (post damping-off), pre-damping-off (no emergence). Data were computed for disease incidence.

Results and discussion

Double-stranded RNA segment pattern

Using the modified method according to Jordan and Dodds (1985) and Valverde *et al.* (1990). dsRNA were detected 2 isolates out of 156 isolates of *Trichoderma* spp. There were three distinct sizes of dsRNA estimated to be one segment of 1000 bp evidenced in isolate TM10 and two segments of 700 and 1100 bp in isolate TM20 (Fig. 1). To confirm the appearing of these dsRNA, total nucleic acid extracts of the fungi were subjected to RNase and DNase digestions. The fragments were resistant to RNase at high ionic strength (2XSSC) and were sensitive to this enzyme at low ionic strength (0.1XSSC) indicating that they were linear dsRNA (Figs. 2A and B). Consistently dsRNA ranging from 2.4 -10 kbp which difference in pattern of 5 patterns were also detected in 12 isolates of *Trichoderma* collected from mushroom farm in Hungari (Antal *et al.*, 2005). Furthermore it was indicated by comparing free dsRNA isolates and dsRNA contained isolates (TM10 and TM20), there were abnormal growth of dsRNA contained isolates including irregularly colonial growth and their growth rate was classified in to medium to low growing group and also showed lower sporulation. Likewise *Fusarium graminearum* contained with dsRNA was characterized and found that dsRNA could effect morphological change of the fungus such as reduction of its growth rate (Chu *et al.*, 2002). Moreover, Van Diepeningen *et al.* (2006) were also found that *Aspergillus* sp. contained with dsRNA was showing low sporulation. Thus it is suggested that dsRNA might impact morphological growth and biological control potential of *Trichoderma*.

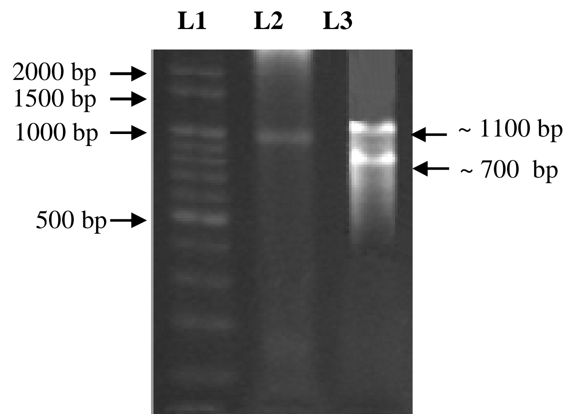

Fig. 1. Agarose gel electrophoresis (1.5%) of dsRNA extracted from *Trichoderma* spp. isolates TM10 (lane 2) and TM20 (lane 3). Arrows indicated the 1000 bp (lane 2) and 700 bp to 1100 bp segments (lane 3). Lane 1 = 100 bp DNA Marker.

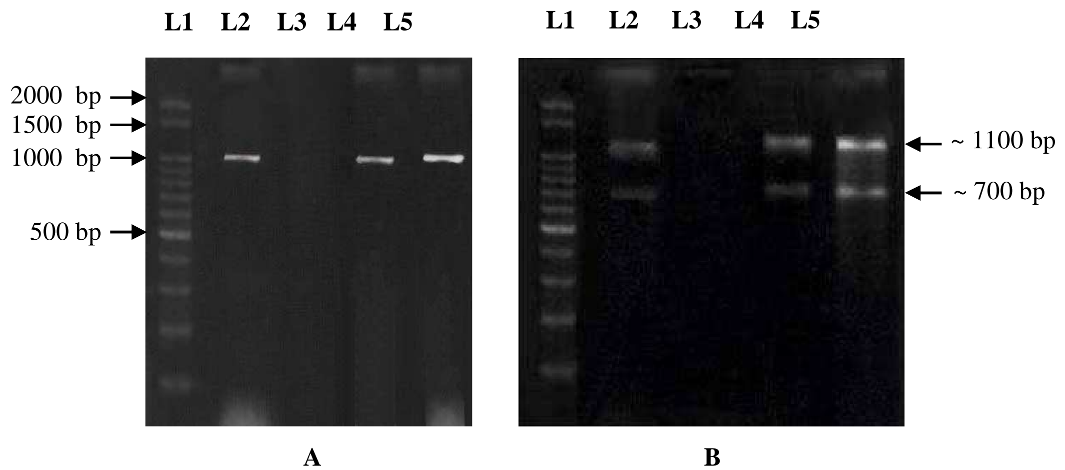

Fig. 2. Agarose gel electrophoresis (1.5%) of dsRNA extracted from *Trichoderma* spp. isolates TM10 (A) and TM20 (B) digested with RNase and DNase. Lane 1 = standard of 100 bp DNA Marker, Lane 2 = dsRNA digested with RNase at high ionic strength, Lane 3 = dsRNA digested with RNase at low ionic strength**,** Lane 4 = dsRNA after digested with DNase and Lane 5 = dsRNA of *Trichoderma* sp.

Elimination of dsRNA

Trichoderma isolates TM10 and TM20 contained dsRNA were subjected to heat treatment. Although dsRNA fragments were still detected but it was found that both fungal isolates TM10h and TM20h gained the higher growth rate and sporulation. Comparison with their parental culture, TM10 and TM20 both TM10h and TM20h isolates were improved their "fluffiness" and growth rate of averaging of 18.40, 24.60, 23.93, and 27.5 mm/day (Fig. 3). Moreover, the heat treated isolates showed more sporulation than untreated isolates with averaged 1.82×10^{10}, 2.60×10^{10}, 3.11×10^{10} and 3.63×10^{10} spore/ml for TM10, TM10h, TM20 and TM20h respectively. The result was consistence with the case of *Rhizoctonia solani* strain PT3-1 and US-9 contained dsRNA which were cultured on PDA supplemented with 10 ppm of cycloheximide and emetine and then subsequent subculture for 10 times but dsRNA were still appeared (Kim *et al.*, 1996). Fekete *et al*. (1995) also reported that subculture event many times could not remove dsRNA from *F. poae*. By other hand, culturing *Nectria radicicola* on PDA supplement with 10 ppm of cycloheximide and 20 ppm of emetine and then removed the fungus to the new PDA resulting no dsRNA detected in *N. radicicola* growth on Czapek dox broth was also reported (Ann and Lee, 2000).

Enzymatic assay

Exochitinase production of *Trichoderma* spp. free dsRNA isolate including T15**,** T42 and T75 and dsRNA contained isolate TM10 and TM20 as well as dsRNA contained isolate after heat treated namely TM10h and TM20h were analyzed. Result showed that there was no quantity significant of enzyme activity among free dsRNA and pre or post heat treated dsRNA contained isolates, amount of enzyme production for 4 days were averaged 59.3×10^{-3}, 54.5×10^{-3}, 60.0×10^{-3}, 53.0×10^{-3}, 54.5×10^{-3}, 50.5×10^{-3} and 56.8×10^{-3} unit/ml for isolates T15, T42, T75, TM10, TM10h, TM20 and TM20h respectively (Fig. 4). With this, Zaldivar *et al.* (2001) reported that there was no different in exo-chitinase production between wild type and mutant strains of *T. aureoviridis.* De-Marco *et al*. (2003) were also reported that production of chitinase N-acetylglusmimnodase and β-1,3- glucanase in three isolate of *Trichoderma* spp. were not difference.

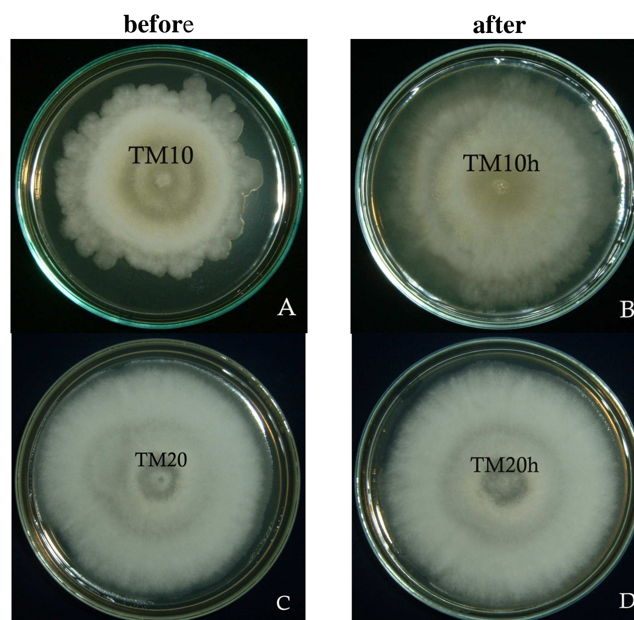

Fig. 3. Colony forming of *Trichoderma* contained with dsRNA (TM10, TM20) before and after heat treatment (TM10h, TM20h). a = TM10, b = TM10h, c = TM20 and d = TM20h.

In-vitro and in-vivo antagonistic activity

In-vitro study of antagonistic activity of *Trichoderma* spp., the free dsRNA, dsRNA contained isolate and heat treated isolate to control *S. rolfsii* using dual culture test showed that T15 gave the most effective isolate to control the pathogen with inhibition percentage of 71.67 %, followed by T75 and T42 (60.79 and 54.93%), all of which were significantly different from dsRNA contained isolates both after and before heat treatment, which TM10 and TM20 showed inhibition percentage of 25.42 and 24.62 % (Fig. 4). Interestingly, it was found that we also heat treated isolates were slightly increased their percent inhibition (28.52 and 26.74%).

In-vivo study, T15 and T75 showed the highest inhibition that could suppress disease incidence of 25%, and followed by free dsRNA isolate. T42 showed diseases incidence of 48.75%. The dsRNA contained isolates as well as dsRNA contained isolates after heat treated could not inhibit the pathogen, therefore disease incidence in pots treated with these isolates were high which averaged 72.50, 66.25, 62.25 and 63.75% respectively. Chu *et al.* (2002) suggested that dsRNA may be related with hypovirulance in fungus. In this study the comparison with free dsRNA isolates and the two dsRNA contained

isolates were decreased in growth rate, spolutation, biological control activity and irregularly colonization both in laboratory and greenhouse.

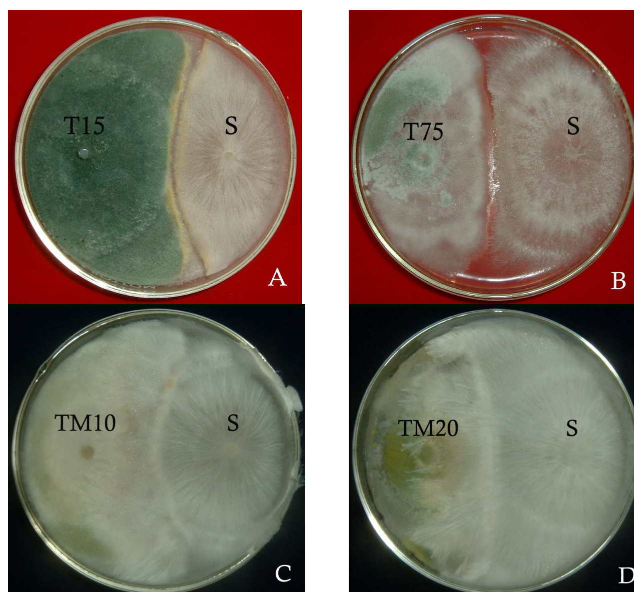

Fig. 4. Dual culture interaction between *Trichoderma* spp.(T) and *Sclerotium rolfsii* (S) on PDA**,** 5 days after inoculation. a = *Trichoderma* spp. isolates T15 (free dsRNA), b = *Trichoderma* isolates T75 (free dsRNA), c = *Trichoderma* spp. isolates TM10 (dsRNA contained) and d = *Trichoderma* isolates TM20 (dsRNA contained).

Conclusions

Two isolates contained dsRNA elements out of 156 isolates of *Trichoderma* spp. were detected. One fragment of 1000 bp and two fragments of 700 bp and 1100 bp dsRNA were detected in isolate TM10 and TM20 respectively. Comparing with free dsRNA isolates, the two dsRNA contained isolates were lower growth rate spolutation, thus there were no differentiation of their exochitinase production. Furthermore, biological control activities both in laboratory and greenhouse to control *S. rolfsii* indicated that the dsRNA contained isolates were lower biological control activities comparing with free dsRNA isolates. For evaluation on dsRNA stability, the contained dsRNA were subjected to heat therapy to eliminate dsRNA. There were slightly improved their mycelial growth and sporulation but the dsRNA were still appeared. The results indicated that dsRNA may influence the efficacy and biological activity of antagonistic fungi including *Trichoderma* spp.. Therefore, the investigation concerned standard method.

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