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Biological Control of *Sclerotium rolfsii* Damping-off of Tropical Pine (*Pinus merkusii*) with Three Isolates of *Trichoderma* spp.

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Abstract: *Trichoderma koningii*, *T. reesei* and *T. harzianum* were tested for their ability to control *Sclerotium rolfsii* damping-off of tropical pine seedlings. Light microscopic observation on dual culture assay showed that the hyphae of all *Trichoderma* isolates could grow parallel to the hypae of *S. rolfsii*. However only *T. reesei* and *T. harzianum* coiled around the hyphae of *S. rolfsii* and formed appresoria and hook-like structures. Both isolates indicated equally high colony growth inhibition toward *S. rolfsii*. In greenhouse experiment, formulated *T. reesei* and *T. harzianum* in alginate beads, applied 4 days before *S. rolfsii* infestation controlled damping-off in pine seedlings effectively. Disease reduction by formulated all *Trichoderma* spp. decreased drastically when applied 4 days after or at the same time of *S. rolfsii* infestation.

Key words: Biological control, *Sclerotium rolfsii*, *Trichoderma*

Introduction

Biological control of plant pathogens is an attractive alternative to the strong dependence of modern agriculture on chemical fungicides, which cause environmental pollution and development of resistant strains. Species of *Trichoderma* have long been known for their capacity to reduce plant diseases caused by fungal pathogens (Baker and Cook, 1974) and some have been tested for biocontrol potential in many field and greenhouse trials.

The properties of an efficient disease control agent, include strong competitive ability, antibiotic production, direct parasitism and lysis (Ayers and Adams, 1981; Baker and Cook, 1974). In our preliminary experiments, 60 different cultures of *Trichoderma* spp. were tested as antagonist against several soil borne pathogenic fungi and found some potent antagonists i.e.: *T. koningii* isolate 1, *T. reesei* isolate 13 and *T. harzianum* isolate 27 (Widyastuti and Sumardi, 1998; Widyastuti *et al.*, 1998a, 1998b, 1999 and 2001a).

In this study, we determined the antagonistic mode of 3 isolates of *Trichoderma* spp. against *Sclerotium rolfsii* Sacc. and evaluated the potential use of the antagonist formulated in alginate beads, for controlling of damping-off of tropical pine (*Pinus merkusii* Jung et. de Vriese) seedlings. Damping-off caused by several species of fungi, i. e. *Sclerotium* sp., *Pythium* sp.,

Fusarium sp. and ***Rhizoctonia*** sp. is a serious problem in pine nurseries since this disease highly disturb the continuity of seedling supply for plantation (Nair and Sumardi, 2000).

Materials and Methods

The experiment was conducted in the Laboratory of Forest Protection, Faculty of Forestry, GMU from January to May, 2002.

Fungal isolates

*T. **koningii*** isolate T1, an effective antagonist against white root-rot pathogen ***Rigidoporus microporus*** which attacked ***Hevea brasiliensis***, was obtained from BPTP Banjar Baru, South Kalimantan, Indonesia. *T. **reesei*** isolate 13 and *T. **harzianum*** isolate T27 were selected isolates collection of Laboratory of Forest Protection, GMU. ***In vitro*** test showed that *T. **reesei*** performed high antagonistic activity against root-rot pathogen ***Ganoderma*** spp. (Widyastuti and Sumardi, 1998; Widyastuti ***et al.***, 1998b; 1999) and *T. **harzianum*** suppressed effectively the growth of *R. **microporus*** isolated from ***Acacia mangium*** (Widyastuti ***et al.***, 1998a). The pathogenic fungus *S. **rolfsii*** was isolated from pine seedling indicating damping-off disease. All of the fungal isolates were maintained on a PDA slant.

Direct confrontation assay for microscopic observation

Dual culture to confront ***Trichoderma*** spp. and *S. **rolfsii*** were established following method developed by Johnson and Curl (1972) with modification. Agar plug cuts containing isolate colony of ***Trichoderma*** each was placed in opposite site, in 9 cm plate containing 7 ml water agar toward agar plug cut of *S. **rolfsii***. Microscopic slide was placed in each plate in between the two plug cuts before water agar was poured. The dual cultures were allowed to grow at 28°C, and the hyphal interaction was observed under light microscope by removing the microscope slide from the plates and staining in 10% (v/v) lactophenol blue for 5 min.

Colony growth inhibition assay

Antagonistic activity of ***Trichoderma*** spp. against *S. **rolfsii*** was evaluated using direct confrontation assay described above, but using 10 ml PDA in each plate with no microscope slide. In this experiment, we also introduced PCNB into the agar medium at final concentration of 100 ppm to compare growth inhibition effectivity between biocontrol agent and chemical fungicide. The experiment was conducted in 5 replicates.

Formulation of ***Trichoderma***. Formulated ***Trichoderma*** in alginate beads were prepared according to Mauperin ***et al.*** (1987) with modification by Widyastuti ***et al.*** (2002a). Briefly, spores suspension of ***Trichoderma*** (final concentration 10^7 spores/ ml) were mixed with sodium alginate and then solidified into beads by adding drops of calcium chloride solution. This results in the encapsulation of spores within beads of alginate gel in diameter sizes 2-3 mm.

Greenhouse test

Five hundred g of autoclave sterilized sand in polyethylene container were used as the basic medium for this test. Fifteen of 10 days old pine seedlings were transplanted to each container and 2 alginate beads/seedling were applied 4 days before, at the same time and 4 days after the time of *S. rolfsii* inoculation. *S. rolfsii* was inoculated by mixing homogenized colony of stock culture thoroughly with sand medium at the final concentration of 200 mg (wet wt.)/kg medium. The stock culture was prepared by growing *S. rolfsii* on PDA for 10 days at 28°C. The treated seedlings were grown under greenhouse condition at 26-32°C. Experimental units were arranged following completely randomized design with 2 replicates. Disease incidence was recorded until no damping-off development on the samples was observed. Damping-off suppression was evaluated using method developed by Hadar *et al.* (1979). Percentage of disease reduction (DR) was calculated according to the following formula:

$$DR = [1 - (DC - DT) / DT] \times 100$$

where DC and DT are percentage of disease in control and treatment, respectively.

Results and Discussion

Observation of hyphal interaction on the confrontation zone indicated that the diameter and the intense of staining of both fungal hyphae were different, so they could easily be distinguished from each other (Fig. 1a-e). Hyphae of *T. reesei* and *T. harzianum* often coiled around the host (Fig. 1a, b), but no coiling was shown by *T. koningii* (Fig. 1c). All *Trichoderma* isolates could frequently grow parallel to the host. *T. reesei* and *T. harzianum* both produced appresoria at the tips of short branches (Fig. 1d) or formed hook-like structures (Fig. 1e). No such structures were formed by *T. koningii*. These findings agreed with the experiment of Elad *et al.* (1983), who observed mycoparasitic activity of *T. harzianum* against *S. rolfsii* and *R. solani* using scanning electron microscopy and fluorescence microscopy.

Table 1: Control of damping-off in pine seedlings caused by *Sclerotium rolfsii*, grown in greenhouse, by three isolates of *Trichoderma* formulated in alginate beads*

	Disease reduction according to *S. rolfsii* inoculation+		
Trichoderma isolates	4 days before	At the same time	4 days after
T. koningii isolate T1	46.7*b*	43.3*ab*	3.3*b*
T. reesei isolate T13	73.3*a*	50.0*a*	23.3*a*
T. harzianum isolate T27	66.7*a*	56.7*a*	6.7*b*

Data in each column which are followed by a common letter are not statistically different (P= 0.05), +Percentage of disease reduction (DR) was calculated in 15-day-old pine seedlings

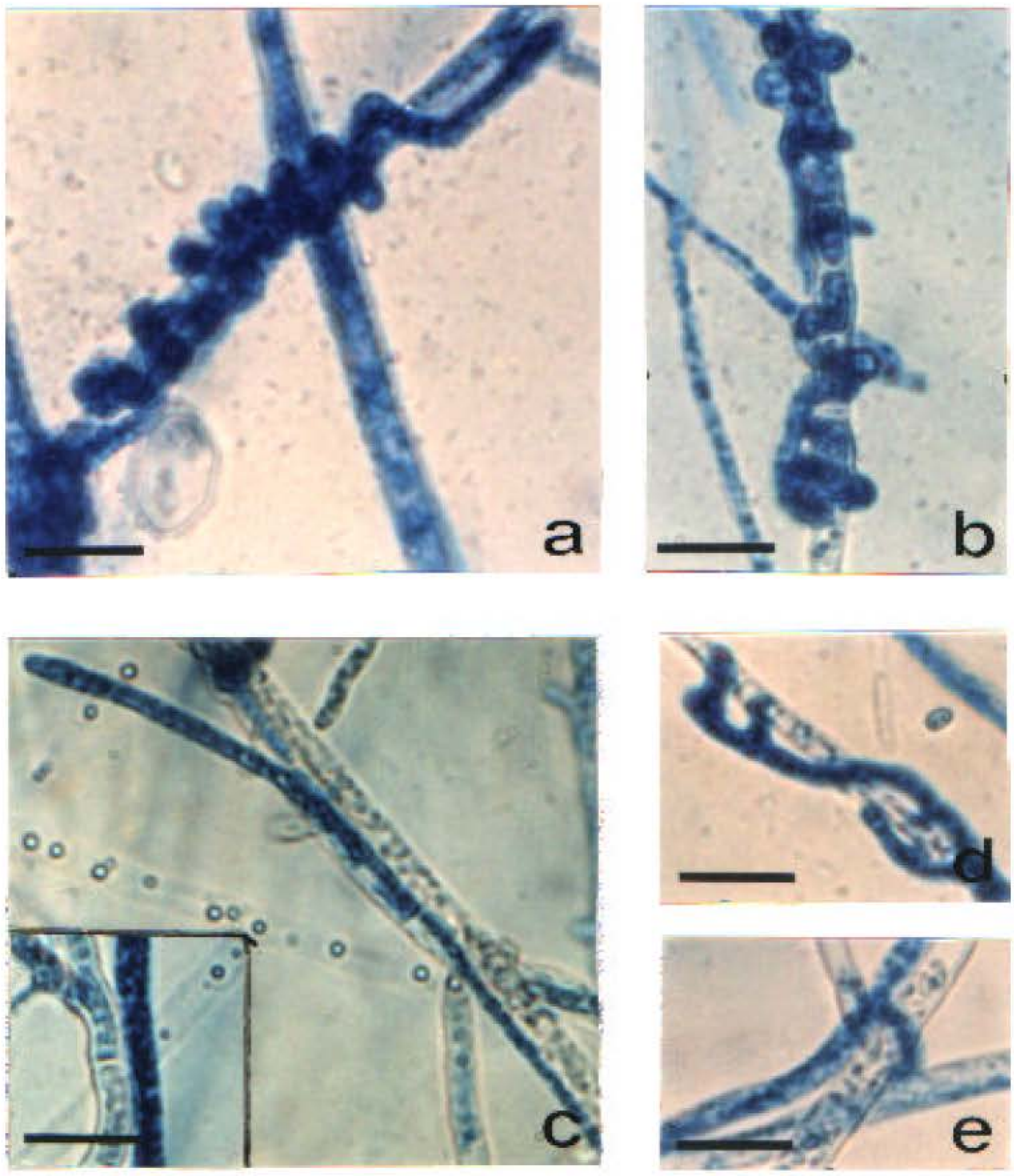

Fig 1: Light microscopy of *Trichoderma* spp. hyphae interacting with that of *Sclerotium rolfsii*. **a** + **b**. Condensed coiling of *T. reesei* and *T. harzianum*, respectively around a hypha of *S. rolfsii*. **c**. Although *T. koningii* could grow parallel to hypha of *S. rolfsii*, no coiling was observed. **d**. Appresorium-like structures, formed by *T. reesei* and also found in *T. harzianum* but was not in *T. koningii*. **e**. Hook-like structure was found in the interaction between hypha of *T. reesei* and *T. harzianum*, in ordered to attach themselves to host mycelium. (Bar represents 10 μm)

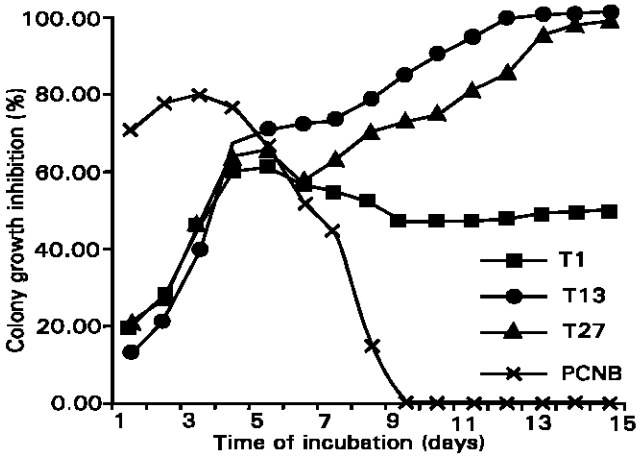

Fig. 2: Growth inhibition of *Sclerotium rolfsii* by *Trichoderma* spp. compared with chemical fungicide PCNB. T1: *T. koningii* isolate T1; T13: *T. reesei* isolate T13; T27: *T. harzianum* isolate T27. The final concentration of PCNB was 100 ppm in 10 ml PDA. The experiment was conducted in 5 replicates

Chet *et al.* (1981) showed that hyphae of *T. hamatum* grew directly towards *R. solani* and indicated that this was not random phenomenon. Upon reaching host hyphae, the antagonistic fungus either coiled around the host or produced appresoria or hook-shaped contact branches. Deacon (1976) hypothesized that coiling occurs when some resistance host is encountered in which the potentially infective hypha may be grown directed towards a more susceptible region. Formation of hyphal coiling around fine glass threads or nylon fibers has been demonstrated (Inbar and Chet, 1992) which suggested that the phenomenon might be more as a response to contact with a surface than a specific response to the presence of a susceptible host hypha.

Different growth inhibitions of *S. rolfsii* by PCNB and by three isolates of *Trichoderma* were observed (Fig. 2). Capabilities of PCNB to inhibit the growth of *S. rolfsii* decreased drastically in 4 days after application. It seemed that the pathogen has capabilities to neutralized inhibition effects of PCNB. *T. koningii* showed lower inhibition compared to *T. reesei* and *T. harzianum* and has tendency to decrease gradually. *T. reesei* and *T. harzianum* showed the best growth inhibition and there was no significant difference (P = 0.05) between the two.

This result was supported by microscopic observations (Fig. 1a-e), where no coiling was found in the hyphal interaction between *T. koningii* and its host. *T. reesei* and *T. harzianum* on the other hand, often coiled around and sometimes produced appresoria.

Harjono *et al.* (2001a, 2001b) recently isolated and characterized endochitinase enzyme from culture of *T. reesei* isolate T13 and found that the enzyme performed *in vitro* antifungal activity against the mycelial of *Ganoderma philippii*. Since the cell walls of *S. rolfsii* are composed of β-

1,3-glucan and chitin (Bartnicki-Garcia, 1973), it was hardly assumed that endochitinase played an important role in the growth inhibition of the pathogen. Mycoparasitic activity of ***Trichoderma*** spp. might be as the result of antibiosis, competition, production of cell wall-degrading enzymes, or combination among these mechanisms. Recent evidence has shown that antibiotics and hydrolytic enzymes are not only produced together but act synergistically in mycoparasitic antagonism (Di Pietro ***et al.***, 1993 and Schirmböck ***et al.***, 1994).

Bell ***et al.*** (1982) and Widyastuti ***et al.*** (1999) showed in paired cultures on agar that a biocontrol isolate which highly effective against one isolate of a pathogen could have performed minimal effect on other isolates of the same pathogen. More evidence supported that this might be related to the pathogen-specificity of antagonistic mechanisms such as antibiotic (Howell and Stipanovic, 1995) and cell wall degrading enzymes (Haran ***et al.***, 1996).

Biological control of damping-off disease by ***S. rolfsii*** achieved by applying ***Trichoderma*** spp. to soil is presented in 1. Application of formulated ***Trichoderma*** 4 days before ***S. rolfsii*** inoculation gave the best disease suppression. Adaptation and establishment of ***Trichoderma*** spp. prior pathogen inoculation might be resulted in increasing their antagonistic capability. ***Trichoderma*** spp. delayed the initiation of symptoms and decreased disease incidence from 73.3 to 46.7%. Disease reduction of ***Trichoderma*** spp. declined drastically when ***Trichoderma*** spp. applied 4 days after ***S. rolfsii*** inoculation. This result agreed with findings of Widyastuti ***et al.***, 2002b that formulated ***Trichoderma*** need adaptation time to be active to inhibit growth of pathogenic fungi.

Like any other organism, a biological control agent is affected by abiotic and biotic factors such as disease pressure and competition from the indigenous microflora. The results imply that time of application might play an important role in considering the method of biological control in the field.

In conclusion, ***T. reesei*** and ***T. harzianum*** provide an effective antagonistic mechanism against ***S. rolfsii***. Both isolates performed high mycoparasitic activity in direct confrontation assay and demonstrated good disease reduction to control damping-off caused by ***S. rolfsii*** in pine seedlings. Results of this experiment could be considered in improving strategy to use ***Trichoderma*** spp. as biocontrol agent.

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