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ORIGINAL PAPER

Biocontrol of *Rhizoctonia solani* AG-2, the causal agent of damping-off by *Muscodor cinnamomi* CMU-Cib 461

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Abstract *Rhizoctonia solani* is a damping-off pathogen that causes significant crop loss worldwide. In this study, the potential of *Muscodor cinnamomi*, a new species of endophytic fungus for controlling *R. solani* AG-2 damping-off disease of plant seedlings by biological fumigation was investigated. In vitro tests showed that *M. cinnamomi* volatile compounds inhibited mycelial growth of pathogens. Among nine solid media tested, rye grain was the best grain for inoculum production. An in vivo experiment of four seedlings, bird pepper, bush bean, garden pea and tomato were conducted. The results indicated that treatment with 30 g of *M. cinnamomi* inoculum was the minimum dose that caused complete control of damping-off symptoms of all seedlings after one month of planting. The *R. solani*-infested soil showed the lowest percentage of seed germination. In addition, *M. cinnamomi* did not cause any disease symptoms. From the results it is clear that *M. cinnamomi* is effective in controlling *R. solani* AG-2 both in vitro and in vivo.

Keywords Damping-off disease · Endophytic fungi · Fungal fumigant · Volatile compound

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Introduction

Rhizoctonia solani Kühn., is an anamorphic soilborne pathogen causing damping-off disease. Disease symptoms include leaf blights, leaf spots, damping-off, rot on roots, shoots and fruits, canker lesions on sprouts and stolons, and sclerotial diseases (Mikhail et al. 2010; Taheri and Tarighi 2012). The teleomorph of this fungus, *Thanatephorus cucumeris* A.B. Frank. Donk., is classified as a basidiomycetous fungus (Sneh et al. 1996). The mycelia or sclerotia of this pathogen can survive during unfavorable environmental conditions for several years within diseased plant material in soil and 13 anastomosis groups (AGs) of *R. solani* have been reported (Carling et al. 2002; Taheri and Tarighi 2012). The host range of *R. solani* is extensive and it causes various diseases on important crop plants of the world including species in the Asteraceae, Braccicaceae, Fabaceae, Poaceae and Solanaceae families (Carling et al. 2002; Ceresini et al. 2007; Mikhail et al. 2010).

Soilborne pathogenic fungi including *R. solani* reduce the quality and quantity of agricultural crops in Thailand (Jetiyanon and Kloepper 2002; Ploetz 2003; Wiwattanapatapee et al. 2004). Using chemical fungicide is the most common method for reducing yield losses caused by fungal pathogens. During the past 10 years, 13–68 % of agricultural crops in Thai markets were contaminated with fungicides according to the Food and Drug Administration of Thailand (Worapong and Strobel 2009). Replacement of fungicides with biocontrol agents is an alternative way to manage plant pathogens, produce safe food and reduce environmental pollution (Barakat and Al-Masri 2005). *Muscodor* species are endophytic fungi that have been isolated from certain tropical trees and vine species in Central/South America, Asia and Australia (Worapong

et al. 2001, 2002; Strobel et al. 2001; Daisy et al. 2002; Sopalun et al. 2003; Ezra et al. 2004; Atmosukarto et al. 2005; González et al. 2009; Suwannarach et al. 2010; Zhang et al. 2010; Kudalkar et al. 2012). An important characteristic of the genus *Muscodor* is an ability to produce volatile organic compounds (alcohols, esters, ketones, acids and lipids) that have biological activities (Strobel et al. 2001; Strobel and Daisy 2003). For this reason, most research has focused on the development of *Muscodor albus* as a biocontrol agent (Mercier et al. 2007; Worapong and Strobel 2009). They have been shown to be an effective biofumigant against fungal fruit decay (Mercier and Jiménez 2004; Mercier and Smilanick 2005) and grain smut fungi (Strobel et al. 2001; Goates and Mercier 2009). A volatile mixture produced by strain CZ-620 was reported to have some nematicidal and insecticidal activity (Riga et al. 2008; Lacey and Neven 2006). Recently, the new endophytic fungus *M. cinnamomi* was found to produce a mixture of volatile organic compounds (Suwannarach et al. 2010).

In this study, we aimed to evaluate the ability of *M. cinnamomi* CMU-Cib 461 as a potential biological agent to control both in vitro and in vivo of *R. solani*, which causes devastating damping-off diseases. The suitable solid media for *M. cinnamomi* inocula production were examined. Therefore, this knowledge could lead to the development of *M. cinnamomi* as a biocontrol agent by mycofumigation, which may replace the toxic fungicides that are currently used.

Materials and methods

Fungal culture

Pathogenic *R. solani* AG-2 was isolated from damaged tomato plants in Chiang Mai Province, Thailand. *Rhizoctonia solani* was preserved on a dry filter paper at 4 °C and was revived by aseptically transferring a piece of the dry filter paper to a potato dextrose agar (PDA, LABSCAN®) plate and grown for 5–7 days in an incubator chamber (25 ± 2 °C). From this stock culture, new cultures were produced by transferring *R. solani* agar plugs (0.5 mm diam) to the center of PDA plates.

Muscodor cinnamomi described previously by Suwannarach et al. (2010), was stored in a 15 % glycerol solution at −20 °C at the Sustainable Development of Biological Resources Laboratory, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand. The *M. cinnamomi* strain CMU-Cib 461 was deposited with the BIOTEC Culture Collection, Pathumthani, Thailand and The Japan Collection of Microorganisms, Japan.

Effect of *M. cinnamomi* volatiles on *R. solani*

A dual culture volatile assay was used in this in vitro experiment. *M. cinnamomi* were inoculated onto PDA and incubated in the incubator chamber for 12 days. A two-compartment plastic plate (92 mm × 16 mm) was loaded with PDA. Then, *M. cinnamomi* was inoculated and grown on one side of the plate for testing. After 3 days, an agar plug (6 mm) was taken from the margin of 5–7 day-old *R. solani* mycelium and placed into the other compartment before sealing with parafilm. The pathogen not exposed to the volatiles of *M. cinnamomi* was used for the control experiment. Colony diameter of *R. solani* was measured after 7 days and the percentage inhibition was calculated. To assess viability of the pathogen, mycelial plugs of *R. solani* were then transferred to fresh PDA and incubated for 7 days. The experiment was repeated twice with five replicates.

Determination of suitable solid medium for *M. cinnamomi* inocula preparation

Nine solid media; barley grain, corn grain, GABA rice grain, kidney bean grain, soybean grain, sorghum grain, mung bean grain, red kidney bean and rye grain, were used in this experiment. The grains were prepared by boiling for 25 min. The boiled grains were 18 mm × 180 mm test tubes to approximately 10 cm depth and autoclaved at 121 °C for 30 min. After cooling, each tube was inoculated with a mycelium plugs (5 mm diam) cut from the periphery of the growing colony and incubated in the incubator chamber for 45 days in darkness. Linear growth of the mycelium was measured every third day, and growth rate was estimated from the linear part of the plot of the length against incubation time (Ohta and Fujiwara 2003).

Plants seedling

Selected locally grown seeds (Chia Tai Co. Ltd., Thailand) of tomato (*Lycopersicon esculentum* Mill.), bird pepper (*Capsicum annuum* L.), garden pea (*Pisum sativum* L.) and bush bean (*Phaseolus vulgaris* L.) were used. Before planting, all seeds were soaked in sterilized distilled water overnight, and the seeds that sunk to the bottom of the vessel were selected for the experiments. Commercial soil (Mae-On Company, Chiang Mai, Thailand) was used in all experiments with a pH of 6.8 soil was autoclaved at 121 °C for 30 min and distributed into each 14 cm × 19 cm pot. Each experiment was conducted in a completely randomized design with four replicates. All experiments were performed in outdoor conditions during October to December 2010, under blue screen netting to protect plants from insect infestation. The temperature varied during incubation from an average high of 21–34 °C, while the

relative humidity ranged from 67 to 72 %. The maximum daily light intensity ranged from 12,040 to 55,000 lux.

Effect of *M. cinnamomi* on *R. solani* in vivo

Three week-old rye grain *M. cinnamomi* was used as inoculum since the fungal mycelium completely covered the surface of rye grain and one plate of 5 day-old mycelium of *R. solani* cultured in PDA. The *M. cinnamomi* inoculum was scraped, cut and minced into the soil.

To determine the minimum dose of *M. cinnamomi* needed to completely control damping-off symptoms, 50 seeds of each plant (tomato, bird pepper, garden pea and bush bean) were grown in pots. Each pot contained 500 g sterile soil with or without one plate of 5 day-old mycelium of *R. solani*. *Muscodor cinnamomi* inocula dosages ranged from 0 to 40 g per pot. The percentage of seeds germinated in each pot was recorded after 10 days.

Four treatments were established to test the effect of *M. cinnamomi* on controlling *R. solani* in vivo. Twenty seeds per pot were used in each treatment. The non-infested control pots contained 500 g sterile soil. The second treatment consisted of 500 g soil and 30 g *M. cinnamomi* inoculum (the minimum dose that caused complete control of damping-off symptoms). The third treatment had 500 g soil, 30 g *M. cinnamomi* inoculum and one plate of *R. solani*. The fourth treatment consisted of 500 g soil and one plate of *R. solani*. After 1 month of incubation, five plants seedlings from each treatment were randomly selected. The root of each selected plant was washed with tap water and the hypocotyl was cut 0.5 cm upper from root. The shoot height, root length and fresh weight of shoots and roots were measured. The experiment was repeated twice with four replicate pots per treatment.

Statistical analyses

All experimental data was analysed by SPSS program version 16.0 for Windows. The data of fungal growth in solid medium was subjected to analysis of variance using Duncan's test ($P < 0.05$). The analysis of variance of seedling emergence was analysed with Fisher's LSD multiple comparison test ($P < 0.05$).

Results

In vitro effect of *M. cinnamomi* volatiles on the growth and viability of *R. solani*

The inhibition effect of the volatile compounds produced from *M. cinnamomi* against the mycelial growth of *R. solani* was tested using a dual culture volatile assay. The results showed that volatile compounds caused 100 % inhibition of *R. solani* mycelial growth. No growth of *R. solani* was observed when transferred to fresh PDA after 1 week of exposure to *M. cinnamomi* volatile compounds.

Determination of suitable solid media

The mycelium growth rates of *M. cinnamomi* in the test tube culture on various media were investigated after 45 days. The fungus significantly grew faster on rye grain (1.86 ± 0.2 mm day^{-1}), followed by GABA rice grain (1.65 ± 0.1 mm day^{-1}) and sorghum grain (1.27 ± 0.1 mm day^{-1}). *M. cinnamomi* did not grow on the soybean and red kidney bean media (Fig. 1).

Effect of *M. cinnamomi* dose range on seed germination

Effects of dosages of *M. cinnamomi* inoculum mixed with soil on the percentage of seed germination of all plant species are shown in Fig. 2. The results showed that increasing the amount of inoculum could decrease the percentage of seed germination of bird pepper bush bean and tomato, with the exception of garden pea.

Effect of *M. cinnamomi* dose range and *R. solani* on seed germination

After 10 days of incubation, seeds failed to germinate, became soft, mushy, turned brown, and decomposed as a consequence of seed infection in the *R. solani*-infested soil treatment. The non-infested control of all plants had the

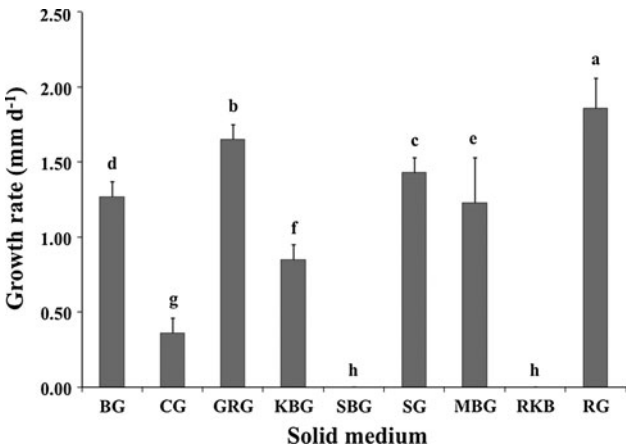

Fig. 1 Growth rate of mycelia of *Muscodor cinnamomi* on different solid media after 45 days. *BG* barley grain, *CG* corn grain, *GRG* GABA rice grain, *KBG* kidney bean grain, *SBG* soy bean grain, *SG* sorghum grain, *MBG* mung bean grain, *RKB* red kidney bean and *RG* rye grain. Data were means of four replicates. *Error bar* at *each point* indicated that ± SD. The *different letters* above *each graph* indicated the significant difference ($P < 0.05$) according to Duncan's multiple range test

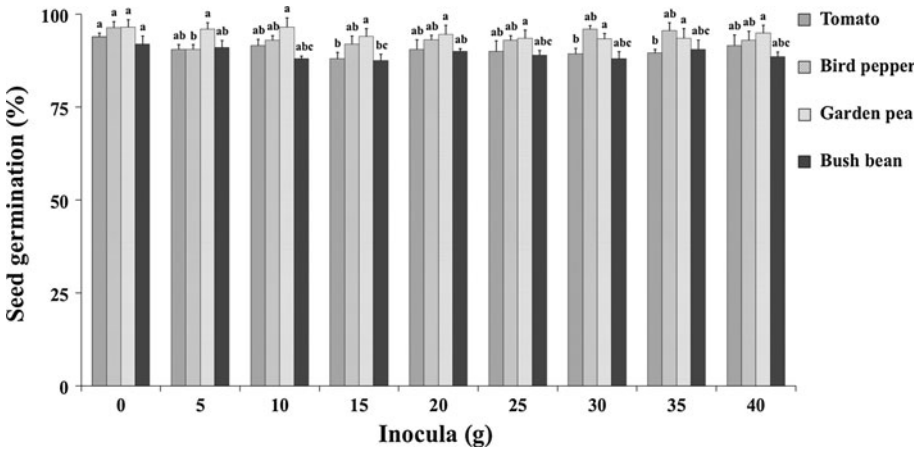

Fig. 2 Percentage of seed germination on different *Muscodor cinnamomi* inocular dose. Data were means of four replicates. *Error bar* at *each point* indicated that ± SD. The *different letters* above *each graph* indicated the significant difference ($P < 0.05$) according to Fisher's LSD multiple comparison test

highest percentage of seeds that germinated (Fig. 3). Treatment with 30 g *M. cinnamomi* with inoculum was the minimum dose that caused complete control of the disease. In addition, the statistical analysis indicated that the percentages of seed germination of bird pepper, bush bean and tomato were reduced after using 30 g inoculum when compared with the non-infested control. This result indicates that *M. cinnamomi* inoculum has phytoinhibitory activity.

Effect of *M. cinnamomi* on plant growth

Seedlings from each experiment were measured for shoot height, root length and fresh weight of shoots and roots. One month after planting, infected seeds had not yet sprouted and seedlings showed damping-off disease in only the *R. solani*-infested soil. The symptoms on tomato and bird pepper seedlings included pale brown stem lesions and soft, water-soaked, collapsed, seedlings that quickly died. Garden pea and bush bean seedlings had symptoms including reddish brown stem lesions, thin tissue, collapsed seedlings and the non-damping-off plants showed stem canker disease. The plant disease symptoms were not found on the non-infested control in the *M. cinnamomi* and *M. cinnamomi* mixed with *R. solani* experiments. The shoot height and fresh weight of all seedlings grown in *R. solani*-infested soil were significantly lower than all other treatments (Fig. 4a, b). The roots of *R. solani*-infested plants were significantly shorter than all other treatments (Fig. 4c, d). The non-infested control had the highest fresh root weight followed by the experiment inoculated with *M. cinnamomi* and *M. cinnamomi* mixed with *R. solani*. This result indicated that *M. cinnamomi* inocula had a slight phytoinhibitory effect on plant growth, but they could not completely control damping-off disease caused by *R. solani* AG-2.

Discussion

The present study demonstrates the biocontrol of damping-off disease caused by *R. solani* AG-2 both in vitro and in vivo using *M. cinnamomi*. The results indicated that *M. cinnamomi* produces volatile compounds which can completely control in vitro mycelial growth of *R. solani* AG-2. This result was similar to the volatile compounds produced by fungi in the genus *Muscodor* which inhibited the in vitro mycelial growth of a wide variety of plant pathogenic fungi,

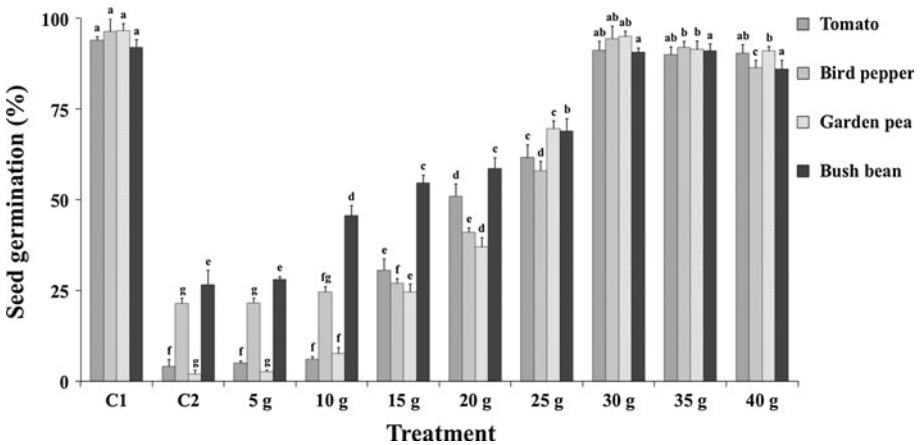

Fig. 3 Percentage of seed germination in the presence of *Rhizoctonia solani* AG-2 with different *Muscodor cinnamomi* inocular dose. C1 = non-infested control and C2 = *R. solani* control. Data were means of four replicates. *Error bar* at *each point* indicated that ± SD. The *different letters* above *each graph* indicated the significant difference ($P < 0.05$) according to Fisher's LSD multiple comparison test

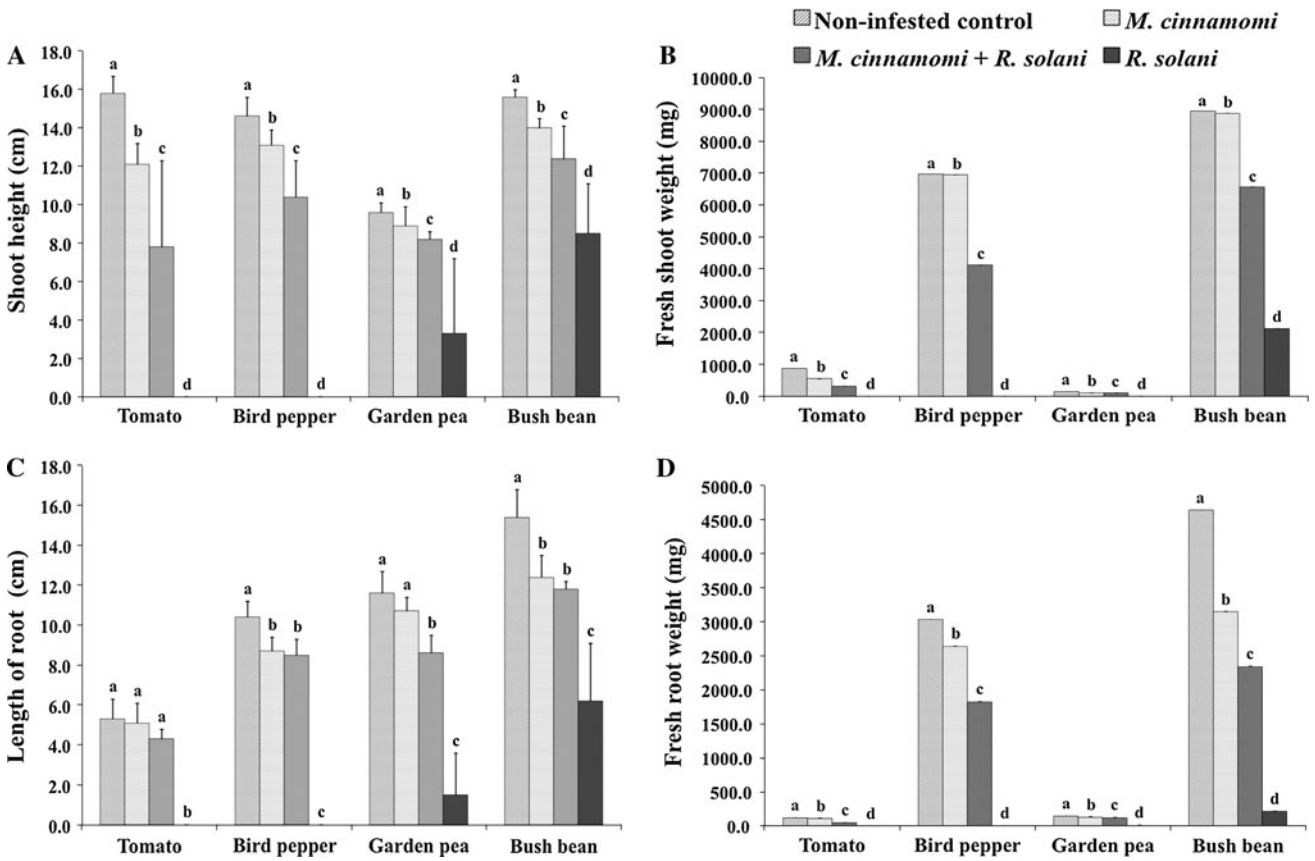

Fig. 4 Shoot and root of seedling in present of *Rhizoctonia solani* AG-2 with 30 g *Muscodor cinnamomi* inocular dose. **a** shoot height of seedlings, **b** fresh shoot weight of seedlings, **c** root length of seedlings and **d** fresh root weight of seedlings. Data were means and *error bar* at *each point* indicated that ± SD. The *different letters* above *each graph* indicated the significant difference ($P < 0.05$) according to Fisher's LSD multiple comparison test

such as *Colletotrichum*, *Fusarium, Penicillium*, *Pythium, Rhizoctonia* and *Verticillium* (Strobel et al. 2001; Worapong et al. 2001, 2002; Ezra et al. 2004; Mitchell et al. 2008; González et al. 2009; Worapong and Strobel 2009; Banerjee et al. 2010; Zhang et al. 2010). Goates and Mercier (2009) reported that volatile compounds from *M. albus* had the potential to reduce in vitro spore germination viability of *Tilletia horrida*, *T. indica* and *T. tritici*. In addition, many fungal species are known to produce volatile compounds, which have prompted research on chemical analysis of some volatile substances common to many fungi (Schnurer et al. 1999; Rapior et al. 2000; Lee et al. 2009). *Trichoderma* spp. produces volatile compounds which inhibit the growth of *Alternaria*, *Colletotrichum*, *Fusarium*, *Rhizoctonia*, *Pythium* and *Sclerotium* (Ajith and Lakshmidevi 2010; Lahlali and Hijri 2010). Some bacterial strains, such as *Bacillius subtilis*, *Pseudomonas chlororaphis*, *P. fluorescens*, *Serratia odorifera* and *Stenotrophomonas maltophilia*, are able to produce organic volatile compounds which inhibit in vitro mycelium growth of *R. solani* (Kai et al. 2007; Sarangi et al. 2010). In addition, previous studies reported that 8 volatile compounds such as aciphyllene; 1-butanol, 3-methyl, acetate; 2-butanone; 1-butanol, 2-methyl; ethyl butyrate; 2-methylfuran; isobutyric acid and tetrahydofuran from *M. albus* showed antibiotic activity (Atmosukarto et al. 2005; Ramin et al. 2005; Mercier et al. 2007). *Muscodor cinnamoni* was found to produce 11 volatile compounds including 1-butanol, 3-methyl, acetate which could inhibit the growth of *R. solani* (Suwannarach et al. 2010). In the future, each volatile compound produced by *M. cinnamomi* and not produced by *M. albus* will be investigated further using biological tests.

In vivo biocontrol of *R. solani* was tested with four plants (bird pepper, bush bean, garden pea and tomato). This study showed that treatment of soil with rye grain culture of *M. cinnamomi* effectively controlled *R. solani* AG-2 damping-off in seedlings of all plant species, suggesting a biological fumigation effect. In previous studies, a greenhouse fumigation experiment of rye grain or ground barley grain culture of *M. albus* and *M. roseus* resulted in successful control of soilborne pathogens, such as *Phytophthora capsici* (Mercier and Manker 2005; Camp et al. 2008), *R. solani*, *Pythium ultimum*, *Aphanomyces cochlioides*, *Colletotrichum coccodes* and

Verticillium dahliae (Stinson et al. 2003; Mercier and Manker 2005; Grimme et al. 2007). Furthermore, Worapong and Strobel (2009) reported that using fresh agar culture of *M. albus* MFC2 incorporated in potting soil infested with *R. solani* controls root rot of kale. In the present study *M. cinnamomi* successfully controlled the effects of *Rhizoctonia* damping-off with 30 g of rye culture of *M. cinnamomi* in 500 g soil. Damping-off control became inconsistent as the dosage went below 30 g. In this study, the amount of inoculum differed from other reports that studied on biocontrol using volatile-producing endophytic fungi. For examples, the effective control of *Rhizoctonia* damping-off of broccoli seedling with rye culture containing 15–30 g of *M. albus* 620 inocula and *Phytophthora* root rot of bell pepper with 25 g mixed and grown in a one liter soilless medium (Mercier and Jiménez 2009). Lahlali and Hijri (2010) reported that 25 g rye grain culture of *Epicoccum nigrum* and *Trichoderma atroviride* in 500 g soilless media could decrease the potato stem disease caused by *R. solani*. Furthermore, Lee et al. (2009) reported that fumigation with 5 g of wheat branrice hull cultures of *Oxyporus latemarginatus* controlled disease development of *Rhizoctonia* root rot on moth orchid by 73 %. Recently, Goates and Mercier (2011) reported that dry rye grain culture of *M. albus* (125 mg per g of infected seed) could control common bunt of wheat caused by *T. caries* in field conditions. This study found that the amount of *M. cinnamomi* inocula affected the percentage of seed germination and growth of tested plants which showed a slight phytoinhibitory activity. This result is similar with Macías-Rubalcava et al. (2010) who reported that the volatile compounds produced by *M. yucatanensis* showed phytoinhibitory activity that inhibited growth of amaranth, barnyard grass and tomato. Furthermore, several reports have shown that rye-cultured *M. albus* completely controlled decay of lemons (Mercier and Smilanick 2005), apples (Mercier and Jiménez 2004), peaches (Mercier and Jiménez 2004; Schnabel and Mercier 2006), grapes (Gabler et al. 2006; Mercier et al. 2005), tomatoes (Freitas et al. 2005) and potatoes (Corcuff et al. 2006).

It is possible that *M. cinnamomi* could be used as a biological control agent to eliminate soilborne diseases without adverse effects to plants by adding active culture to soil before planting. With the phasing out of methyl bromide and environmental concerns about other chemical alternatives, considerable efforts are being made to find environmentally friendly alternatives to chemical fumigation. Further study on the optimal solid media and techniques of inoculum production will be studied with a focus on low cost and easily managed methods. This knowledge will allow for *Muscodor*-based biological control agents in field conditions on a commercial scale in Thailand.

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