
Application of endophytic fungi from para rubber tree as high ability in inhibitors of against *Phytophthora botryosa*, the pathogen of leaf-fall disease in para rubber tree

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Piyaboon, O., Thunyamada, S., Numsiripongpan, W., Pattayanan, K., Wattanagetkul, M., Ophawatanasina, T., Tantithama, N. and Upala, W. (2013) Application of endophytic fungi from para rubber tree as high ability in inhibitors of against *Phytophthora botryosa*, the pathogen of leaf-fall disease in para rubber tree. Journal of Agricultural Technology 9(5):1179-1187.

Para rubber is one of the most important economical plants of Thailand. However, many farmers encounter the reduction of both quality and quantity of rubber latex caused by *Phytophthora* leaf fall and pot rot. This study aimed to evaluate endophytic fungi in para rubber roots and their possible antagonistic activities against pathogenic fungus, *P. botryosa*. Roots of para rubber were collected from 5 random areas of farmer's field in Ban Kha District, Ratchaburi Province. Endophytic fungi were isolated from surface sterilized root tissues. A total of 107 isolates were found and 22 isolates of there were identified by dual culture test to have the efficiency in inhibiting *P. botryosa* more effective than 0.1% Metalaxyl. The four most effective isolates this series from namely S2P81-1, S2P12-1, S2P31-2, and S2P13-1 were then further tested for their antagonistic ability against the fungi *P. botryosa* on leaves of para rubber. These four endophytic fungi isolates were also tested for their efficiency on RIMM 600 rubber trees, which was expressed as the percentage of inhibition of the para rubber growth. The results have shown that endophytic fungi (S2P13-1 and S2P31-2) as well as 0.1% Metalaxyl caused similar percentages of inhibition of the fungi *P. botryosa* growth on para rubber tree.

Keywords: endophytic fungi, rubber latex, dual culture, antagonistic activity

Introduction

Para rubber is one of the most important economic trees in Thailand. Normally, para rubbers are planted in the southern part of Thailand where temperature and moisture are appropriate for their growth. However, moisture can indirectly cause damage to the tree as the moisture is the chief factor contributing to the development of fungi, especially *Phytophthora botryosa*,

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resulting in diseases in para rubber (Churngchow and Rattanasarn, 2001). The symptoms of the disease can be conspicuously observed at the petiole; the diseased trees demonstrate the dark brown or black scar along with the length of the petiole. The scar, an entrance of the pathogen, is attached by the miniscule drops of water. Furthermore, the disease can be observed by lightly flicking the leaves; consequently, unlike the leaflets of healthy trees, those of the infected ones are easily fall down. Occasionally, the dark brown or black, bruised wounds appear on the leaves, and the sizes of wounds are varied. If the moisture is at high level, the white fungi will efficaciously develop and cover the sheath. This infected sheath will be decayed on the tree and not naturally fall down; subsequently, the source of the pathogen is occurred. These microorganisms require the water to reproduce, and therefore, this disease is efficaciously prevailed in the cool weather and rainy, highly continued moisture condition. Nowadays, the agriculturalists usually employ the chemicals, such as Metalaxyl and fosetyl-AI, to prevent the plants from the fungi. The most often used method is squirting the chemicals to prevent the trees from pests but the customers recently become more aware of the remaining chemicals in the produce and environment. Thus, alternative choices are created in order to substitute the use of chemicals, and one of those options is biological control. The agriculturalists became more interested in and approved of this method. Moreover, they realized the benefits of testing the efficiency of selected fungi which may lead to an effective biological method to inhibit *P. botryose*, the pathogen of leaf fall disease.

Therefore, there is an attempt to use endophytic fungi, another kind of fungi found in trunk and branch tissues of para rubber trees, to control the *P. botryosa* biologically. Endophytic fungi do not cause any harm to rubber trees; instead they live with the tree protooperatively – the trees provide endophytic fungi with food and habitation while in return the fungi produce secondary metabolites or hormones which are beneficial to them (Hyde and Soyong, 2008). Also, endophytic fungi which are extracted from root, trunk, or leaf tissue are capable of inducing immunity against harmful microorganism (Chanway, 1997), as well as creating substances that destroy any harmful bacteria or molds (Brem and Leuchtman, 2001). For example, endophytic fungi extracting from *Oryza sativa*, a type of Indian rice, can inhibit the growth of three pathogens, namely *Chaetomium globosum*, *Penicillium chrysogenum* and *Streptomyces* sp. (Naik *et al.*, 2007).

The purpose of this study was to carry out in the field condition in order to search an alternative option to decrease the damages from the destroying of diseased fungi in rubber tree. The biological control method is to lessen the contamination in environment and the danger of the pesticides to farmers.

Materials and methods

Strains of fungus *Phytophthora botryosa*

Phytophthora botryosa isolated from infected rubber trees, was a gift from Office of Research and Development of Plant Protection, Department of Agriculture, Bangkok, Thailand.

Characteristics of the fungus *Phytophthora botryose*

Phytophthora botryosa was grown and maintained in Potato Dextrose Agar (PDA) under appropriate conditions. Its hyphae and spores' microscopic structures were studied by using Lactophenol Cotton Blue (LCB) stain.

Isolation of endophytic fungi from the roots of para rubber trees

Plant materials of para rubber trees were collected from 5 random areas in a farmer's field, Ban Kha District Ratchaburi Province, Thailand. Mature healthy plant roots were collected from different parts of para rubber trees. Samples were transported in a cooler, stored overnight at 4 °C and processed on the following day. Para rubber roots were cut diagonally into approximately 7 to 8 centimetres. The root samples were surface sterilized by procedures as previously described (Coombs and Franco, 2003). The surface sterilized root segments were evenly spaced in Petri dishes (9 cm diameter) containing PDA medium (added with streptomycin 0.3 mg/ml) and incubated at 28 °C for 7 day. The Petri dishes were monitored every day to check the growth of endophytic fungal colonies from the root segments. The hyphal tips, which grew out from leaf segments were isolated and identified using the colors of their hyphae and spores. Pure cultures were then transferred to PDA Petri dishes free of antibiotics and cultivated at 28 °C for 14 days on PDA plates.

Determination of antagonistic ability of endophytic fungi against the fungi *P. botryosa* in laboratory

Antagonistic ability of endophytic fungi against *P. botryosa* was carried out on PDA medium using the dual culture test (Evans *et al.*, 2003). Five millimeter diameter mycelia plugs of *P. botryosa* was placed in corners of culture plates and incubated for 4 days at 28 °C. After that five millimeter diameter mycelia plugs of each of the endophytic fungi was inoculated at the center of each PDA plate and incubated at 28°C for 5 days. Two different experiments were employed as the control groups: the first control group (Rpc)

used only *P. botryosa*, and the second control group (Rnc) used 0.1% Metalaxyl (5 grams of Metalaxyl from Farm Agro Chemical Company in 100 sterlite water) instead of endophyte fungi. When endophytic fungi grew more than 2.5 cm, the radius of *P. botryosa* (Rt) in experimental groups and control groups were measured. This was performed in *triplicate*. The inhibition rates were then calculated from the obtained radii observed in *P. botryosa* (Rt) groups and compared with the first control set by using the equation : The percent inhibition of radial growth (RI) = [(Rpc-Rt)/Rpc] x100.

Determination of antagonistic ability of endophytic fungi against the fungi P. botryosa on leaves of Para rubber

Base on our results showing that endophytic fungi have an ability to inhibit *P. botryosa*, the four most effective endophytic fungi isolated against *P. botryosa* namely S2P81-1 S2P12-1 S2P31-2 and S2P13-1 were collected and tested on leaves of Para rubber. The experiments were conducted in a randomized complete block design with three replications of each treatment. The sterilized cock borers were used to pierce and pick 7-days-old *P. botryosa* and put it on leaf of Para rubber for 3 days at room temperature. The spores of four most effective endophytic fungi was counted by hemacytometer and the solution was diluted with sterile water until the concentration is 10⁸ spores per milliliter and 0.1% Metalaxyl (5 grams of Metalaxyl from Farm Agro Chemical Company in 100 sterlite water) were sprayed on tested leaves. Level of symptom seen on leaves of para rubber were recorded every day for 3 days, The Disease Severity Index, and the percentage of the inhibition of *P. botryosa* activity were evaluated. Levels of the symptom were level 0: normal leaf, no disease, more than 80% of leaf area was normal, and leaf stalk was still green; level 1: leaf showed the symptom, there were spots on leaf, and 60-80% of leaf area was normal and healthy leaf areas; level 2: leaf showed the symptom, there were many spots on leaf, the spots became brown, and 40-60% of leaf area was normal; level 3: leaf was dead, and less than 40% of leaf area was normal. The disease severity index was calculated from the following equation:

$$\text{The Disease Severity Index} = \frac{\text{Total (Level of the symptom x Number of disease plant)}}{(\text{Highest level of the symptom x Number of plant})}$$

The percentage of inhibition of *P. botryosa* activity was determined as the following equation:

$$\text{Percentage of inhibition of } P. \textit{ botryosa} = (R1-R2)*100/R1$$

Where: R1 = The Disease Severity Index of the second control set

R2 = The Disease Severity Index of experiment set having antagonistic microorganisms

Preparation of endophytic fungi to inhibit fungi *P. botryose* in field condition

The fungi *P. botryose* was cultivated PDA for 7 days; then, the cork borer was used to chip hypha for 50 corks. These corks were placed in 50 milliliters of water, and blended this composition with 5 grams of rice haute and 95 grams of sand (referred as “Oat meal sand”). The mixture was incubated in the dark area at 28°C with the relative humidity of 40% for 5 days.

The solution of four most effective endophytic fungi was prepared in sterile water to a final concentration is 10^8 spores per milliliter.

Determination of the endophyte in inhibiting *P. botryosa*, causing leaf fall disease in para rubber in field condition

The efficiency of four most effective endophytic fungi in inhibiting *P. botryose* was tested in the field condition. There were 6 experiments in each set of trial. The RRIM 600 para rubber trees were implanted by the budding method obtained from the garden in Rayong province. Before experiment, were allowed the seedlings to acclimize to new environment by planting them in soil and left under the 50% shading net for 1 week. The experiment was consisted of into 3 control groups and 4 experimental groups (N= 6 para rubber trees, each). The control groups 1 and 2 were sprinkled with 50 ml distilled water, and control group number 3 was sprinkled with 0.1% Metalaxyl solution. The experimental groups, endophytic fungi isolate S2P81-1 S2P12-1 S2P31-2 and S2P13-1 were sprinkled with 50 ml of endophytic fungi isolate S2P81-1 S2P12-1 S2P31-2 and S2P13-1, respectively. After one day, 100 gram of prepared *P. botryosa* fungi mixed with Oat meal sand was added into the soil of each seedlings for the control groups 2-3 and experimental groups, endophytic fungi isolate S2P81-1 S2P12-1 S2P31-2 and S2P13-1. The sprinkling of endophytic fungi and 0.1% Metalaxyl were repeated every 3 days. Thereafter, daily observation for 20 days was conducted by counting the leaves on the plants, color changed leaves, spotted leaves, number of spots on leaves, characteristics of leaf stalks, and appearance of roots (only on the last day). Then, determined the severity of disease into 4 levels according to procedure from Cirulii and Alexander (1996). The index of severity was calculated by using the evaluated score of leaves from each experimental group according to method of (McKinney, 1923), then used the index of severity to calculate percentage of disease suppression in Para rubber seedlings.

Statistical analysis

All three experiments were conducted in a randomized complete block design with three replications of each treatment. In each experiment, the parameters assessed were expressed as mean \pm SD. Percentage of inhibition of *P. botryosa* and the index of severity to calculate percentage of disease suppression in para rubber were calculated for the mean value and standard deviation. Statistical differences between studied groups were assessed by One way-ANOVA, followed by multiple comparisons utilizing Duncan's multiple range test. Statistical analysis was performed by SPSS statistics software (version 15.0, Window). A $P < 0.05$ was considered to be statistically significant.

Results and discussions

Efficacy of endophytic fungi from the roots of Para rubber trees against P. botryosa in laboratory

From the separation of endophytic fungi from roots of Para rubber, a total of 107 isolates were isolated. Overall, the percentage of the inhibition caused by endophytic fungi isolate varied from 2.82% to 86.71%. Of these, 22 isolates have been shown to have significant ability to inhibit the growth of *P. botryosa*. The percentages of the inhibition of *P. botryosa* by these 22 isolates were greater than 0.1% Metalaxyl. These suggested that each isolate has different ability to inhibit the growth of *P. botryosa*. It may be due that endophytic fungi also had the competition mechanism where they are growing faster than pathogens, and then they could compete for the food and area. They may have the hyperparasites and predation mechanisms by growing within pathogen cells and getting food from those cells. That caused the pathogen cells died. Finally, they could induce disease resistance to the plants by activating the plants to have resistivity against the pathogens (Cook and Baker, 1983). Finally, they could induce disease resistance to the plants by activating the plants to have resistivity against the pathogens.

Efficacy of endophytic fungi against P. botryosa on leaves of Para rubber

After selecting four most effective isolates namely S2P81-1, S2P12-1, S2P31-2, and S2P13-1, they were tested on leaves of Para rubber comparing with 0.1% Metalaxyl, in which this the concentration is normally used by farmers. On day 3, the percentage of the inhibition of *P. botryosa* activity on leaves of Para rubber produced by four most effective isolates S2P81-1,

S2P12-1, S2P31-2, and S2P13-1 were 100%, 76.5%, 70.6%, and 94.1%, respectively in Table 1.

Table 1. The percentages of the inhibition of *P.botryosa* (%) on leaves of Para rubber produced by selected endophytic fungi isolates as compared with 10% Metalaxyl

Endophytic fungi	Percentages of the inhibition (%)*
S2P1-81	100.0±0.0a
S2P12-1	76.5±0.5b
S2P 2-31	70.6±0.3c
S2P1-13	94.1±0.8a
10% Metalaxyl	64.7±0.2d

*Means±SD in the same column followed by a common letter are not significantly different (P>0.05).

There was no statistical difference between the efficiencies of the percentage of the inhibition of *P. botryosa* of each isolate. The efficiencies of these four endophytic fungi isolates was also comparable with 0.1% Metalaxyl. The results conformed to Piyaboon *et al.* (2011) found that the efficiencies of *Trichoderma harzianum* CB-Pin-01 and *Trichoderma harzianum* PM51 were conducted to suppress the disease symptom on leaves of para rubber.

Efficiency of endophytic fungi against P. botryosa in field condition

The efficiency of 4 isolates of high efficient endophytic fungi, S2P1-81, S2P1-12, S2P2-31, and S2P1-13, on Para rubber tree was expressed as the index of severity of disease. The index of severity on day 20 of endophytic fungi isolates S2P1-81, S2P1-12, S2P2-31, and S2P1-13 were 16.7%, 0.00%, 11.1%, and 16.7%, respectively. The index of severity, then was converted into percentages of suppression of endophytic fungi isolate S2P1-81, S2P1-12, S2P2-31, and S2P1-13, which were 75.0%, 100.0%, 83.3%, and 75.0%, respectively, as shown in table 2.

Table 2. Percentage of controlling disease (%) on para rubber produced by selected endophytic fungi isolates as compared with 10% Metalaxyl

Endophytic fungi	Percentage of suppressing disease (%)*
S2P1-81	75.0±1.8c
S2P12-1	75.0±1.5c
S2P 2-31	83.3±2.5ab
S2P1-13	100.0±0.0a
10% Metalaxyl	80.04±2.7b

* Means±SD in the same column followed by a common letter are not significantly different (P>0.05)

The efficiency of endophytic fungi (S2P13-1 and S2P31-2) and 0.1% Metalaxyl had similar. Endophytic fungi from the roots of para rubber trees were to protect para rubber against *P. botryosa* both in vitro and greenhouse conditions. Based on previous report that endophytic fungi are able to protect *Crinipellis pernicioso* the causal agent of Witches' Broom Disease of Cacao (Rubini *et al.*, 2005). Endophytic fungi isolated from root para rubber have relation and interaction between *P. botryosa*. Endophytic fungi have been recognized previously, reflecting differences in evolutionary relatedness, taxonomy, plant hosts, and ecological functions (Stone *et al.*, 2004). Comparison efficiency of four endophytic fungi were inhibited *P. botryosa* in laboratory and in greenhouse. Endophytic fungi isolate S2P1-81 was able to inhibit completely *P. botryosa* in laboratory or on leaves, but reduced the symptoms in greenhouse. We may suggest that endophytic fungi isolate S2P1-81 was low efficiency in greenhouse plants because the competition between endophytic fungi isolate S2P1-81 and indigenous community associated to the host seedlings (Rubini *et al.*, 2005).

Conclusion

Endophytic fungi in roots of para rubber were separated in total of 107 isolates. Among them, 22 isolates was found to have percentage of inhibition to *P. botryosa* more than 80%, and 4 most high efficient in suppression were S2P1-81, S2P1-12, S2P2-31, and S2P1-13. All of there four endophytic fungi isolates were tested for the efficiency in controlling *P. botryosa* fungi on para rubber. It was found that there was no significant difference in efficiency in inhibiting *P. botryosa* among there four endophytic fungi isolates. Their efficiencies were also comparable to that produced by 0.1% Metalaxyl, same concentration used by farmers. When the efficiency of the four endophytic fungi isolates in suppression of *P. botryosa* fungi on Para rubber tree was tested, it was shown that the isolates S2P13-1 and S2P31-2 had similar efficiency to 0.1% Metalaxyl. These results found in the present study suggest that these selected endophytic fungi isolates have high efficiency to suppress the growth of *P. botryosa* which caused severe disease in para rubber. This provides potential for development of bio-product from endophytic fungi to be an alternative non-chemical fungicides for farmers to use in Para rubber plantation.

Acknowledgements

This research was financially support by The Thailand Research Fund and Department of Biology, Mahidol Wittayanusorn School, Nakhon Pathom, Thailand for supporting equipment in this research. The authors wish thank Professor Dr. Sompon Wanwimolruk, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand for the critical reading of the manuscript.

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(Received 27 June 2013; accepted 31 August 2013)