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35	Abstract	White root disease causing by *Rigidoporus* sp. is a severe problem that decreases latex productivity and can even cause mortality of rubber trees. With the aim to control biologically this disease, antifungal actinobacteria were isolated from rhizospheric soils of some medicinal plants cultivated in Thailand. Among all isolated actinobacteria, an isolate TM32 exhibited distinctive antagonistic activity against the fungus. Cell-free culture broth of the isolate showed median effective dose (ED_{50}) of 2.61 ml l^{-1} (equal to 1.19 g l^{-1} of metalaxyl). The isolate was also able to solubilize phosphate and to produce chitinase (enzyme activity = 0.093 ± 0.004 U ml^{-1}), siderophore (average clear zone, 11.75 ± 0.96 mm) and indole-3-acetic acid (54.00 ± 1.00 µg ml^{-1}). Application of biocontrol starters produced by this isolate in nursery stage of rubber trees farming showed greater suppression of the disease than direct use of its biocontrol agents. The biocontrol starters also enhanced growth of the rubber trees by increasing their heights. This might be due to the persistent growth of the isolate by using the organic substrate remaining in soil, which could later antagonize fungal pathogens through colonization at the rhizosphere and immunization of the rubber trees. The isolate revealed phylogenetically related to *Streptomyces sioyaensis* supported by 99 % similarity of 16S rRNA gene sequences. We concluded that application of the biocontrol starters produced by this *Streptomyces* isolate would be an alternative approach for sustainable control of soil-borne fungal invasion in long-term rubber tree farming.
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Utilization of rhizospheric *Streptomyces* for biological control of *Rigidoporus* sp. causing white root disease in rubber tree

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Abstract White root disease causing by *Rigidoporus* sp. is a severe problem that decreases latex productivity and can even cause mortality of rubber trees. With the aim to control biologically this disease, antifungal actinobacteria were isolated from rhizospheric soils of some medicinal plants cultivated in Thailand. Among all isolated actinobacteria, an isolate TM32 exhibited distinctive antagonistic activity against the fungus. Cell-free culture broth of the isolate showed median effective dose (ED_{50}) of 2.61 ml l^{-1} (equal to 1.19 g l^{-1} of metalaxyl). The isolate was also able to solubilize phosphate and to produce chitinase (enzyme activity = 0.093±0.004 U ml^{-1}), siderophore (average clear zone, 11.75±0.96 mm) and indole-3-acetic acid (54.00±1.00 μg ml^{-1}). Application of biocontrol starters produced by this isolate in nursery stage of rubber trees farming showed greater suppression of the disease than direct use of its biocontrol agents. The biocontrol starters also enhanced growth of the rubber trees by increasing their heights. This might be due to the persistent growth of the isolate by using the organic substrate remaining in soil, which could later antagonize fungal pathogens through colonization at the rhizosphere and immunization of the rubber trees. The isolate revealed phylogenetically related to *Streptomyces sioyaensis* supported by 99 % similarity of 16S rRNA gene sequences. We concluded that application of the biocontrol starters produced by this *Streptomyces* isolate would be an alternative approach for sustainable control of soil-borne fungal invasion in long-term rubber tree farming.

Keywords *Hevea brasiliensis* · Soil-borne fungal pathogen · Biocontrol · Actinobacteria · Antifungal activity

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Introduction

Rubber trees, known also as pará rubber trees (*Havea brasiliensis* (Willd. ex Adr. de Juss) Muell. Arg.), are an important economic crop in southern Thailand (Kaewchai et al. 2009). There are a number of plants that can produce natural latex but *H. brasiliensis* is a major contributor. It contributes 99 % of the natural latex in the world market for the manufacture of natural rubber products. These products include automobile tires, gloves, condoms and clothes (Nandris et al.

1987a; Rose and Steinbüchel 2005). More than 70 % of the natural latex was produced from Southeast Asia mainly in Thailand, Indonesia and Malaysia (Rose and Steinbüchel 2005). In 2010, the export market of this natural latex from Thailand was increased 78 % over the previous year with the gross income of USD8.1 billion, and it seems to be increasing year by year (Business Report Thailand 2011). Productivity of the natural latex depends significantly on the health of the rubber trees and their planting season. The rubber trees take approximately 5–6 years to grow to be ready to harvest their latex (Evueh et al. 2011). During this long term cultivation together with preference of wet weather, there is a high risk of invasion by soil borne fungal pathogens (Kaewchai et al. 2009). Fungal infection especially at the root system of the rubber trees can cause a severe reduction in latex productivity and may lead to their mortality (Wilhelm 1973; Nandris et al. 1987a, b; Evueh and Ogbebor 2008). This infection problem critically influences the investment for rubber trees farming.

Among the soil borne fungal pathogens, a Basidiomycete, *Rigidoporus* sp. (formerly called as *Fomes lignosus*) causing white root disease is a worldwide serious problem that can deplete the productivity of the natural latex and lead to massive death of the rubber trees (Wilhelm 1973; Nandris et al. 1987a, b; Jayasuriya and Thennakoon 2007; Kaewchai et al. 2009; Ogbebor et al. 2010). The fungal pathogen lives and remains in soil for a long period of time by production of white mycelia (1–2 mm thickness) that can adhere to the surface of the root bark where their rhizomorphs develop to several metres length in the soil nearby. Healthy rubber trees around can be infected directly by this fungal network or even by root contact between the trees themselves. Infection mechanisms of this fungus involve release of extracellular enzymes that are able to decay the wood and collar of host plants, while other parts of the plants' root system are invaded by its hyphae (Nandris et al. 1987a; Nicole and Benhamou 1991; Kaewchai et al. 2009). Where the infection network develops, it causes much damage over a wide area. Therefore, healthy and immunized rubber tree seedlings are required prior to cultivation.

Chemical treatment is a choice to reduce severity of the disease and to prevent invasion of pathogenic fungi. However, this protection may be expensive. Moreover, awareness of chemical use in agriculture is an important issue due to both toxic accumulation in food and effects on biodiversity of useful soil organisms (Wilhelm 1973; Ogbebor et al. 2010; Evueh et al. 2011). Biological control is an attempt to reduce chemical use and pollution in agriculture. It can be applied to control white root disease in rubber trees but it is limited especially in large scale plantations when the infection happened while the trees are mature. Only antagonistic fungi like *Trichoderma* spp. (Jayasuriya and Thennakoon 2007; Kaewchai and Soytong 2010), *Lentinus squarrosulus* (Idwan et al. 1992), *Chaetomium* spp. and *Aspergillus niger* (Kaewchai and Soytong 2010) have been investigated as biological agents for suppression of the white root disease in rubber trees. However, the field application of these fungi has yet been unclear. Use of potential rhizospheric actinobacteria for the biological control of this disease has not yet been studied. In rhizosphere, these actinobacteria are some of the dominant soil bacteria; they are fast-growing, and play an important role in protecting their associated plants from soil borne pathogens. In addition, most of the actinobacteria can produce plant growth hormones and exchange soil mineral nutrients, which further enhance growth and improve plant health (Tokala et al. 2002; Vasconcellos and Cardoso 2009; Loqman et al. 2009).

In order to reduce the use of chemical fungicides and to sustain rubber tree farming with a biological approach, the main objective of this work is to isolate potential actinobacteria from rhizospheric soils of medicinal plants for biological control of *Rigidoporus* sp. causing white root disease in rubber trees. Both in vitro modes of action for inhibiting the fungal growth and plant growth promoting abilities of some distinct actinobacterial isolates obtained were evaluated. Either direct application of biocontrol agents or biocontrol starters produced by the actinobacteria were tested as alternative approaches for suppression of the disease at the nursery stage of rubber tree farming, and the optimal approach is proposed and discussed in this article.

Materials and methods

White root disease causing fungus

A fungus, *Rigidoporus* sp. causing white root disease was kindly provided by the Rubber Research Center, Surat Thani province, Thailand. Pathogenicity of the fungus was confirmed by inoculation and re-isolation from living roots of rubber trees, where the disease symptoms were observed. The inoculation was done

after growing the fungus on 100 g of sterilized wheat grains at 30 °C for 15 days, and the whole fungal culture was inoculated at the bottom of a pot before planting rubber trees. Budded rubber trees (cultivar RRIM600) at an age of 8–10 months were purchased from Wangthong district, Phitsanulok, Thailand and used throughout this work. The fungus was also isolated from infected roots of diseased rubber trees grown at the Rubber Research Center. Briefly, the infected roots were washed by running tap water to remove soil particles for 10 min and washed twice with sterilized distilled water. Small pieces of the roots were cut and put on Potato dextrose agar (PDA) (Difco^{TM}, MD, USA) plus chloramphenicol and streptomycin at the final concentration of 50 mg l^{-1} each. The agar plates were then incubated at 25 °C until the fungal mycelia were growing out of the roots. Isolated fungi were sub-cultured until they were pure cultures and identified preliminarily based on morphological observations compared to known *Rigidoporus* sp. The observations were done microscopically and macroscopically. All isolates of *Rigidoporus* were allowed to grow on PDA and maintained at 4 °C for further use.

Isolation of actinobacteria from rhizospheric soils

Rhizospheric soils were collected from the field of medicinal plants: turmeric (*Curcuma longa*) and ginger (*Zingiber officinale*) in Chiang Mai province, Thailand. The soils were air-dried at ambient temperature prior to oven-drying at 120 °C for 1 h (Tamura et al. 1997). Dried soil samples were then pretreated with 1.5 % (*w*/*v*) phenol solution (Hayakawa et al. 2004). The supernatant of this solution was serially diluted by sterilized distilled water before spreading on Humic acid-vitamin agar (Hayakawa and Nonomura 1987) plus nystatin and cycloheximide at the final concentration of 25 and 10 μg ml^{-1}, respectively. The agar plates were incubated at 30 °C for a month. All visible actinobacterial isolates were selected, sub-cultured and purified individually on Hickey-Tresner (HT) agar (Atlas 1946), which were further maintained at 4 °C as a working stock and in 20 % (*v*/*v*) glycerol at −20 °C for a long term storage.

Determination of generic abundance of rhizospheric actinobacteria

Total actinobacterial isolates obtained from the same amount of each rhizospheric soil were counted. These rhizospheric actinobacteria were classified using some phenotypic data (morphological characteristics of mycelia and spores, and cell wall diaminopimelic acid (DAP)) into three groups including *Streptomyces*, *Micromonospora* and unknown genus. The actinobacterial isolates were grown on International *Streptomycetes* project medium II (ISP2) agar (Atlas 1946) at 30 °C for a number of days, allowing them to form mature spores. Morphological observations were carried out microscopically under a light microscope (Olympus BH-2, Japan). DAP isomeric analysis was done following a procedure described by Hasegawa et al. (1983).

Screening of antifungal actinobacteria

Antifungal activity of all rhizospheric actinobacteria was assayed against *Rigidoporus* sp. by dual culture technique. Each actinobacterial isolate was grown by streak-plate method on ISP2 agar at 30 °C for 7 days, where an agar plug (5 mm Ø) of the fungus grown previously on PDA at 30 °C for 7 days was placed 4 cm from the actinobacterial streak line. The dual culture plate was incubated at 30 °C, where the antifungal activity was observed everyday. Size of any apparent inhibition zone at the interaction area of both microorganisms was measured (approximately a week after incubation). Antagonistic effects of the actinobacteria on the physiology of the fungal mycelia at the inhibition zone were observed microscopically. Different antifungal levels were determined by different sizes of the inhibition zones (Table 1).

Phylogenetic analysis of the most potent actinobacterial isolate

The most potent actinobacterial isolate that exhibited the largest inhibition zone against *Rigidoporus* sp. was selected. Its genomic DNA was extracted following the method described by Hopwood et al. (1985). The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using a pair of universal primers: 27 F (5′-AGAG TTTGATCMTGGCTCAG-3′) and 1525R (5′-AAGG AGGTGWTCCARCC-3′) (Lane 1991). PCR product was purified using QIAquick PCR Purification Kit (Qiagen) and sequenced by Macrogen Inc. (Seoul, Korea). The gene sequence obtained was compared with all accessible sequences in GenBank database using BLASTN software. Multiple sequence alignments and phylogenetic tree were carried out using the neighbour-

t1.1 **Table 1** Actinobacteria isolated from rhizospheric soils of medicinal plants

t1.2 Rhizospheric soil	Actinobacteria (no. of isolate)	Total	No. of bioactive isolate showing different sizes of inhibition zone				No. of inactive isolate
t1.3			>20 mm	16–20 mm	11–15 mm	<11 mm	
t1.4 Ginger (*Zingiber officinale*)	*Streptomyces* (82)	127	0	1	5	3	73
t1.5	*Micromonospora* (8)		0	0	2	1	5
t1.6	Other genera (37)		0	1	3	6	27
t1.7 Turmeric (*Curcuma longa*)	*Streptomyces* (48)	82	1	0	4	9	34
t1.8	*Micromonospora* (8)		0	1	1	2	4
t1.9	Other genera (26)		0	0	3	3	20
t1.10 Total		209	1	3	18	24	163

joining method (Saitou and Nei 1987) in the Molecular Evolutionary Genetics Analysis (MEGA) program version 4 (Tamura et al. 2007), where the bootstrap confidence levels were computed based on 1000 replications.

Evaluation of in vitro antifungal activity

Some antifungal actinobacteria (giving relatively large (≥16 mm) inhibition zones) were evaluated in their modes of antifungal action including productions of antifungal agents, siderophores and cell wall degrading enzymes. Briefly, the antifungal actinobacteria were grown individually in 100 ml of ISP2 broth at 30 °C with shaking at 120 rpm for 7 days, while their cell-free culture broths (CCBs) were prepared by filtering through 0.20-μm filter paper (Millipore Corporation, Benford, MA). Antifungal activity of the CCBs against *Rigidoporus* sp. was confirmed by agar well diffusion assay. Inhibition capacity (% inhibition) of the CCBs was evaluated using dry weight of fungal biomass (drying at 55 °C for 3 days) after growth at 30 °C for 20 days on PDA supplemented with each CCB (at variant concentrations of 10, 20, 30, 40 and 50 % (*v*/*v*)) that was covered with cellophane. The dry weight of fungal biomass after growth in the same conditions on PDA without CCB supplement was used as a control. The CCB giving the highest % inhibition was evaluated by its median effective dose (ED_{50}) for inhibition of fungal growth compared with known concentrations of a chemical fungicide, Metalaxyl (Local trademark, Samut Prakan, Thailand).

The production of siderophores was assayed using Chrome azurol S (CAS) agar (Schwyn and Neilands 1987). The CCBs were individually applied to both iron-restricted and iron-rich CAS agars. Appearance of a yellow to orange halo on the blue CAS agar according to the production of siderophores was observed, where the diameter of the halo was measured.

Production of some cell wall degrading enzymes (*β*-1,3-glucanase, cellulase, chitinase and protease) was assayed by growing the antifungal actinobacteria at 30 °C for 7 days on a set of agar media supplemented with respective substrates of the enzymes tested. The agar media used were Minimal agar (% (*w*/*v*) of yeast extract, 0.2; KH_2PO_4, 0.1; $MgSO_4$, 0.5; agar powder, 1.5) plus 0.5 % (*w*/*v*) of carboxymethyl cellulose (CMC) for glucanolytic and cellulolytic activities, Chitin agar (Malviya et al. 2009) for chitinolytic activity, and Skim milk agar (Atlas 1946) for proteolytic activity. Staining with an aqueous solution of 1 mg ml^{-1} Congo red for 15 min followed by washing with 1 M NaCl solution was applied for observations of glucanolytic and cellulolytic activities (Teather and Wood 1982). The enzyme activities of CCBs derived from the antifungal actinobacteria were also determined, while these CCBs were prepared after the actinobacterial growth at 30 °C by shaking at 120 rpm, for 7 days in 100 ml of the liquid media mentioned previously, except for the chitinase activity that Chitin-peptone medium (pH 6.8) (Malviya et al. 2009) was used instead. The *β*-1,3-glucanase and cellulase activities of the CCBs were measured by dinitrosalicylic acid method (DNS) Cattelan et al. (1999), while the chitinase activity was measured following the protocol described by Malviya et al. (2009). The protease activity was measured

according to the protocol described by Yang and Huang (1994).

Evaluation of plant growth promoting potentials

Some plant growth promoting potentials of the antifungal actinobacteria such as productions of indole-3-acetic acid (IAA) and ammonia together with capable of solubilization of phosphate, were assessed. The ability to produce IAA was evaluated following the method described by Bano and Musarrat (2003). Briefly, one ml of the same CCB as used for antifungal activity test was mixed with 2 ml of Salkowski's reagent, where the appearance of a pink colour indicates IAA production. Optical density at 530 nm was used to estimate the level of IAA produced compared with an IAA standard curve.

A full loop of each isolate of the actinobacteria was inoculated into 10 ml Peptone water (Atlas 1946) and incubated at 30 °C for 48 to 72 h. Nessler's reagent (0.5 ml) was added into the culture broth, where the colour brown turning to yellow was determined as positive ammonia production.

The ability of the actinobacteria to solubilize inorganic phosphate was assessed using Pikovskaya's agar (pH 7) (Nautiyal 1999). The actinobacteria were inoculated on the agar by spot technique, and incubated at 30 °C for 7 days. Appearance of a clear zone reflecting the removal of phosphate was determined as a positive phosphate solubilization. Size of the clear zone was measured by subtracting the diameter of the actinobacterial colony.

Suppression of white root disease in nursery stage of rubber trees

The budded rubber trees, cultivar RRIM600 as mentioned previously, were screened based on similarities of fertility and size. Agricultural loamy soil was used naturally for planting the rubber trees in all treatments. Five treatments were carried out: 1) a control of planted rubber trees untreated (R), 2) a pathogenic treatment of R invaded by the fungus (RF), 3) a chemical treatment of RF with metalaxyl (RFM), 4) a supplemental treatment of R with the biocontrol agents (RBa), and 5) a treatment of RF with the biocontrol agents (RFBa). The soil invaded with the fungus was prepared by inoculating the whole volume of *Rigidoporus* sp. growing on 100 g of sterilized wheat grains at 30 °C for 15 days, onto the bottom of a pot before planting the rubber trees. The chemical treatment was prepared by mixing the soil with metalaxyl at the final concentration 1 mg ml^{-1} per 1 kg of soil. The biocontrol agents were prepared by growing the most potent actinobacterial isolate in 200 ml of ISP2 broth at 30 °C with shaking at 120 rpm for 7 days. Whole culture broth was used as the biocontrol agents, which was then mixed well with the soil at the final concentration of 6.67 % (*v*/*v*). Ten rubber trees were used individually for each treatment. All treatments were carried out for 5 months under the same weather condition with approximate 10 h of daylight period, and were watered every 3 days using 100 ml of tap water per pot per time. Survival rate (%) of the rubber trees in each condition was calculated. Healthy score of the survival trees was observed and determined in 3 levels; weak = 1, medium = 2, firm = 3 (for stem and branch), and yellow or burned = 1, yellowish green = 2, green = 3 (for leaf). The total score was summarized and calculated as a percentage.

The biocontrol agents were also developed in the forms of biocontrol starters. Four different starters were prepared using different holding organic materials, including: starter Bs1) paddy: rice bran (3:1 *v*/*v*), starter Bs2) sorghum grain: agricultural loamy soil (3:1 *v*/*v*), starter Bs3) solely sorghum grain, and starter Bs4) solely agricultural loamy soil. Most prepared materials were sterilized once at 121 °C for 20 min before use, except for the soil that was sterilized twice at the same condition. The sterilized materials (160 cm^3) were inoculated with 50 ml of the biocontrol agents and incubated at 30 °C for 30 days. These prepared biocontrol starters were individually mixed with the soil at the final concentration of 10 % (*v*/*v*). These soils were used to plant the rubber trees under the same planting conditions described previously for the evaluation of the biocontrol agents. Five rubber trees were used individually for each treatment. Cumulative increased-height of survival rubber trees was recorded by subtracting initial height at the start of the experiments, and used to determine potential of each biocontrol starter.

Statistical analysis

Comparisons of multiple means with standard deviation (SDs) obtained from any treatments were performed using the SPSS 16.0 computer program (SPSS, Chicago IL, USA), with one-way analysis of variance (ANOVA) and Tukey's *post hoc* tests at different significant levels (P). F-distribution (F) value and its appropriate

significant level derived from each comparison were indicated elsewhere of this article.

Results

Phytopathogenicity of *Rigidoporus* sp. causing white root disease in rubber trees

Rigidoporus sp. caused the disease in nursery rubber trees within week 6–8 after it was introduced into the soil for planting the rubber trees. Symptoms of the disease began with the occurrence of yellowish leaves (week 7) followed by burned leaves (week 10). Infected nursery rubber trees died at week 12–13, while the rhizomorph of the fungus covers all areas of fibrous and lateral roots of the trees. The macroscale observation of the disease symptoms is shown in Fig. 1.

Actinobacteria from rhizospheric soils of medicinal plants

A total of actinobacteria (209 isolates) was isolated from rhizospheric soils of medicinal plants (Table 1). Most of the actinobacteria (61 %) were found in the rhizospheric soils of ginger. On the basis of preliminary phenotypic classification, *Streptomyces* was the dominant group of actinobacteria found in rhizospheric soils of both ginger (65 %) and turmeric (58 %). Most of the actinobacteria (78 %) were inactive against

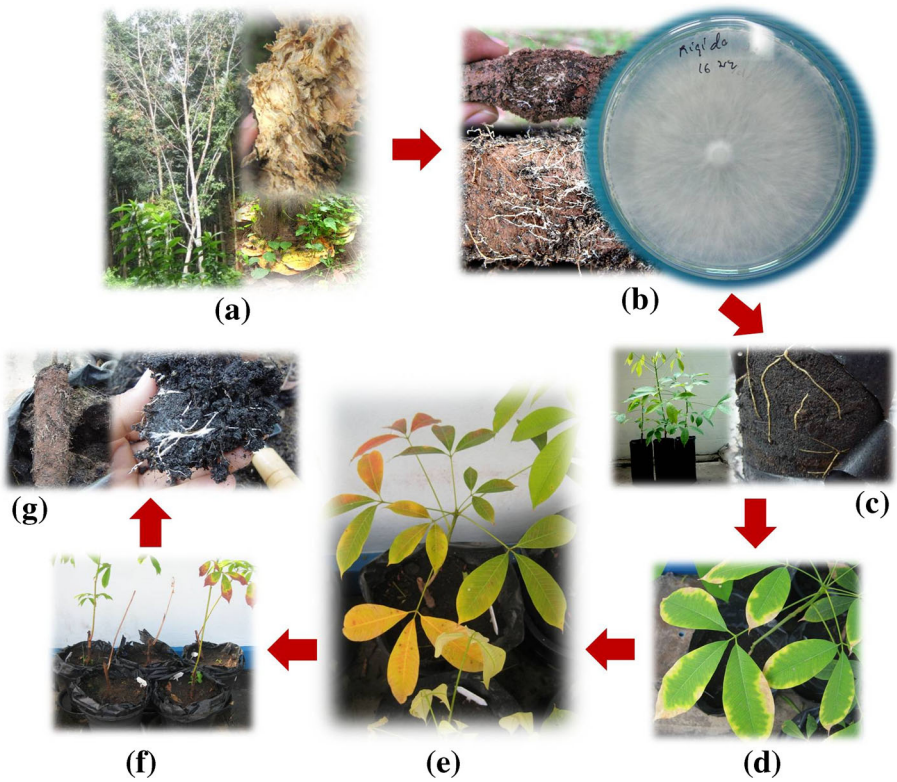

Fig. 1 The macroscale observation of the white root disease caused by *Rigidoporus* sp. Dead rubber tree after infection by the fungus for several months standing in farm without leaf (**a-***left*), while the fungus formed fruit body at the base of the rubber tree (**a-***right down*) where the white fungal mycelia was observed in the inner wood of this infected zone (**a-***right top*). The root of this rubber tree was colonized with the white fungal mycelia, which was later isolated as a pure culture of the fungus (**b**) (see also Materials and Methods). Greenish leaves with firm root system were determined as healthy rubber trees (**c**). After inoculating the fungus into the soil for cultivation of rubber trees, the leaf color turned to yellow at week 7–8 (**d**) and be burned at week 10–12 (**e**). Most leaves had fallen at week 12–13, determined as the dead rubber trees (**f**). At the base and root of the dead rubber trees is a covering with white mycelia of the fungus (**g**)

t2.1 **Table 2** Modes of action against *Rigidoporus* sp. and plant growth promoting potentials of distinct antifungal actinobacteria isolated from rhizospheric soils of medicinal plants

t2.2 Modes of action and plant growth promoting potentials	Actinobacterial isolate			
t2.3	TM32	GN12	GN15	GN20
t2.4 Antagonistic activity [inhibition zone (mm)]	24.00±1.00	18.33±0.58	16.00±1.00	16.67±0.58
t2.5 Siderophore production [halo zone (mm)]	11.75±0.96	8.00±1.41	18.50±1.29	5.25±0.50
t2.6 β-1,3-glucanase	0^a, 0^b	20.33 ± 0.58^a, 0.83 ± 0.05^b	42.50 ± 1.32^a, 1.16 ± 0.12^b	0^a, 0^b
t2.7 Cellulase	0^a, 0^b	20.33 ± 0.58^a, 0.0097 ± 0.0021^b	42.50 ± 1.32^a, 0.71 ± 0.13^b	0^a, 0^b
t2.8 Chitinase	22.67 ± 0.58^a, 0.093 ± 0.004^b	0^a, 0^b	17.17 ± 0.76^a, 0.060 ± 0.002^b	17.50 ± 0.50^a, 0.050 ± 0.004^b
t2.9 Protease	0^a, 0^b	0^a, 0^b	0^a, 0^b	0^a, 0^b
t2.10 Production of indole-3-acetic acid (μg ml^{-1})	54.00±1.00	34.33±1.53	51.67±1.53	72.50±2.18
t2.11 Production of ammonia	–	–	+	+
t2.12 Phosphate solubilization [solubilizing zone (mm)]	8.83±0.76	0	5.50±0.50	15.17±0.76

Actinobacteria isolated from rhizospheric soils of turmeric and ginger, were coded TM and GN, respectively. All bioassay results except for production of ammonia are presented as mean±standard deviation (SD), while the alphabetic indices refer to [a]; clear zone of enzyme assay on solid agar (mm) and [b]; enzyme activity of CCB (U mg^{-1}). The production of ammonia was reported with positive (+) and negative (−) tests. All tests were done in triplicate

Rigidoporus sp., while four actinobacterial isolates (TM32, GN12, GN15 and GN20) showed distinct antifungal activity by giving relatively large inhibition zone (≥16 mm). Only two (TM32 and GN12) out of these four isolates belonged to the *Streptomyces* group. Isolate TM32 originating from the rhizospheric soil of turmeric was determined as the most potent actinobacterial isolate, giving the largest inhibition zone (24.00±1.00 mm) against the fungus (Table 2). The antifungal activity of this isolate at the inhibition zone revealed that the isolates produced antifungal substances causing the fungal mycelial wilt (Fig. 2). The isolate was classified into the genus *Streptomyces* on the basis of its morphology to form long spiral chain spores (Fig. 3) and its cell wall comprised of LL-DAP and glycine, which is the cell wall chemotype I of the genus *Streptomyces* (Lechevalier and Lechevalier 1970). In addition, a total 1452 nucleotides sequence of its 16S rRNA gene revealed it was closely related to *Streptomyces sioyaensis* (DQ026654) supported by 99 % sequence similarity (Fig. 4).

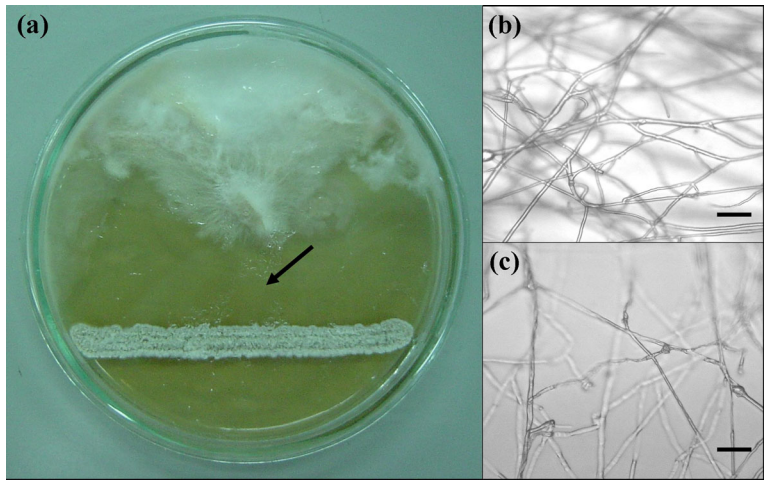

Fig. 2 Dual culture assay of *Rigidoporus* sp. and *Streptomyces* sp. TM32. The assay was done on ISP2 agar incubated at 30 °C for 7 days (**a**), while the arrow points at the interaction zone. Morphology of the fungal mycelia grown on the agar without isolate TM32 (**b**) was compared with the wilt fungal mycelia taken from the interaction zone of the dual culture (**c**). Bars= 10 μm

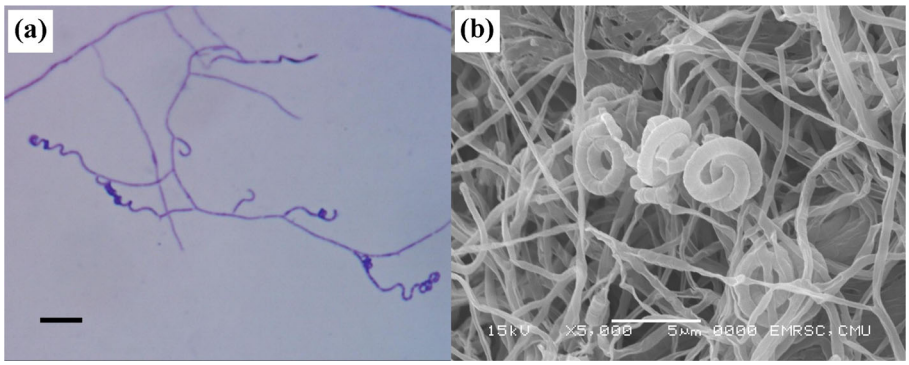

Fig. 3 Morphology of *Streptomyces* sp. TM32. Isolate TM32 was grown on HT agar at 30 °C for 10 days. Then, the aerial mycelia and spores of isolate TM32 were observed and photographed under light (**a**) and scanning electron (**b**) microscopes. Bars=5 μm

Antifungal activity and plant growth promoting potentials of the rhizospheric actinobacteria

Modes of action and some plant growth promoting potentials of the four distinct actinobacterial isolates are shown in Table 2. They inhibited the growth of *Rigidoporus* sp. with different antifungal levels determined by the sizes of inhibition zones. They could also produce siderophores and at least one cellulolytic enzyme, but no production of protease was observed. Isolate TM32 showed the highest chitinase activity (0.093±0.004 U mg^{-1}) followed by isolates GN15 and

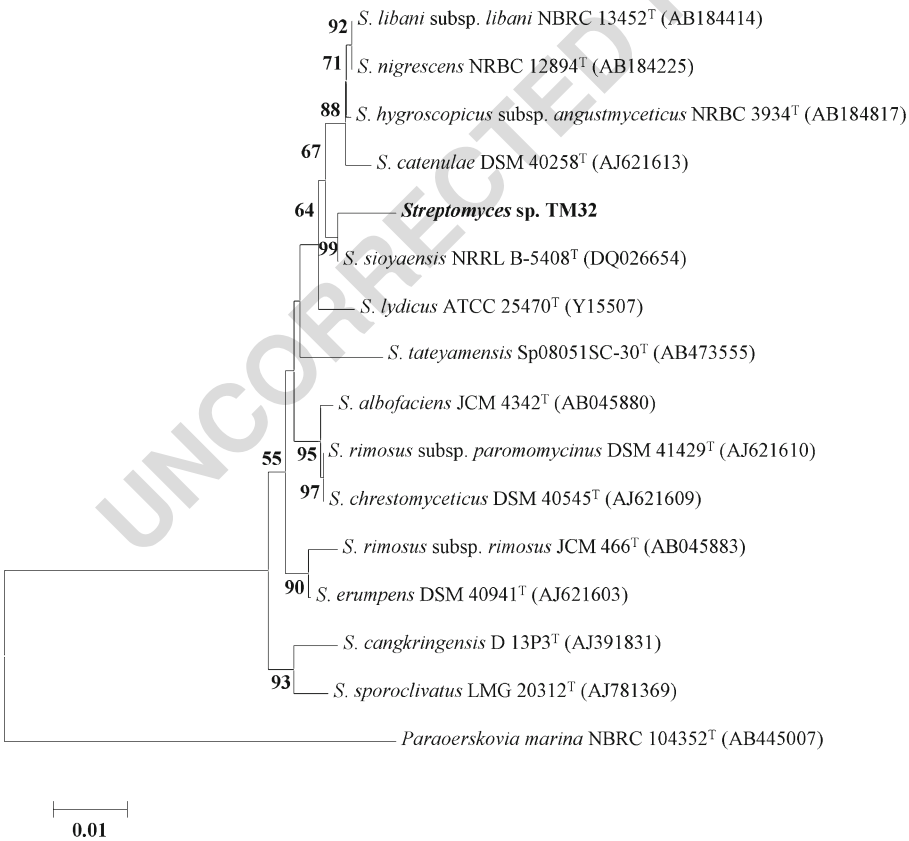

Fig. 4 Phylogenetic tree based on the 16S rRNA gene sequence of *Streptomyces* sp. TM32. The gene sequence (1452 nucleotides without gap) of isolate TM32 (in *bold*) was aligned and compared to the available sequences in GenBank database (accession numbers are given in parentheses). The out-group used was *Paraoerskovia marina*. The bootstrap values greater than 50 % of 1000 replications are shown as percentages at branching points of the tree, where the bar is 0.01 K_{nuc}

GN20. In vitro antifungal activities of the CCBs derived from the four isolates are shown in Fig. 5a. The CCB derived from isolate TM32 could inhibit the fungal growth nearly 100 %, while its inhibition values were not different significantly across the different concentrations tested ($F_{(4,\ 15)}$=0.62, P=0.0001). At the same concentration (10^{F1}, 20^{F2}, 30^{F3}, 40^{F4} and $50\%^{F5}$ (v/v)) compared, the CCB of isolate TM32 showed significantly greater % inhibition than those of other CCBs derived from other isolates ($F1\text{-}5_{(3,\ 12)}$= 346.92, 117.29, 361.88, 426.98 and 256.16, P= 0.0001). Median effective dose (ED_{50}) of this CCB calculated by logistic regression equation was 2.61 ml l^{-1} or 0.26 % (v/v) compared with a standard curve of the known concentrations of metalaxyl (Fig 5b). Some plant growth promoting potentials of the four isolates are shown in Table 2. All isolates could produce IAA, while the highest production was found by isolate GN20 (72.50± 2.18 μg ml^{-1}). The isolate GN20 was also the greatest phosphate solubilizer, giving the largest solubilizing zone (15.17±0.76 mm). Only the isolates GN15 and GN20 were able to produce ammonia.

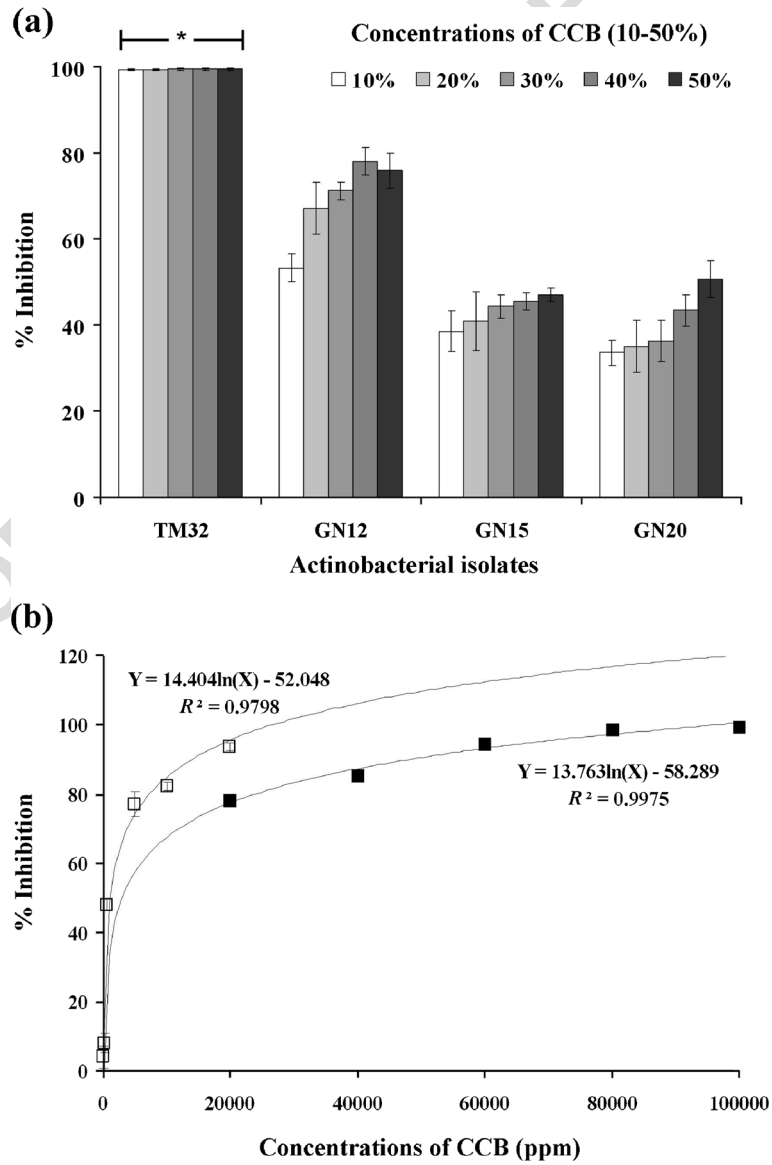

Fig. 5 In vitro antifungal activity of actinobacteria isolated from rhizosphere soils of turmeric (TM) and ginger (GN). Percent inhibition (mean±SD) was determined by dry weight of the fungal biomass grown on PDA plus different concentrations of CCB obtained from the actinobacteria (see also Materials and Methods) (**a**), while the asterisk refers to no difference of means compared by ANOVA at P=0.0001. Logarithmic regression equation of the linear relation between the different concentrations of CCB obtained from *Streptomyces* sp. TM32 (*black square*) was compared to the chemical treatment using the known-concentration of metalaxyl (*white square*) (**b**). All experiments were done in four replications

Utilization of *Streptomyces* sp. TM32 for suppression of white root disease in rubber trees

Uses of both biocontrol agents and biocontrol starters in nursery stage could suppress the disease causing by *Rigidoporus* sp. (Fig. 6). The highest survival rate (100 %) and healthy score (91 %) of rubber trees were observed in the supplemental treatment (RBa) (Fig. 6a). The chemical treatment (RFM) showed equal both survival rate (40 %) and healthy score (16 %) to the pathogenic treatment (RF). The biocontrol agents could suppress the disease caused by the fungus (RFBa) to approximately 20 % greater extent than the chemical treatment. Modification of biocontrol agents in the forms of biocontrol starters (Bs1-4) was carried out using different holding materials for sustaining the growth of isolate TM32. No dead rubber tree was observed when any biocontrol starters were applied. Potential of each biocontrol starter was determined by cumulative increased height of the rubber trees grown in fungal invaded soil (RF) treated with each biocontrol starter (Fig. 6b). Among all biocontrol starters, starter Bs3 prepared with sorghum grain was the optimal one for suppression of the disease, revealing the highest cumulative increased-height at week 14 of the experiments. However, the statistical comparison demonstrated that the potential of this starter was not significantly different from starter Bs2, starter Bs4 and chemical treatment but significantly different from starter Bs1, the control and the pathogenic treatment ($F_{(6,\ 28)}$=10.66, P=0.01).

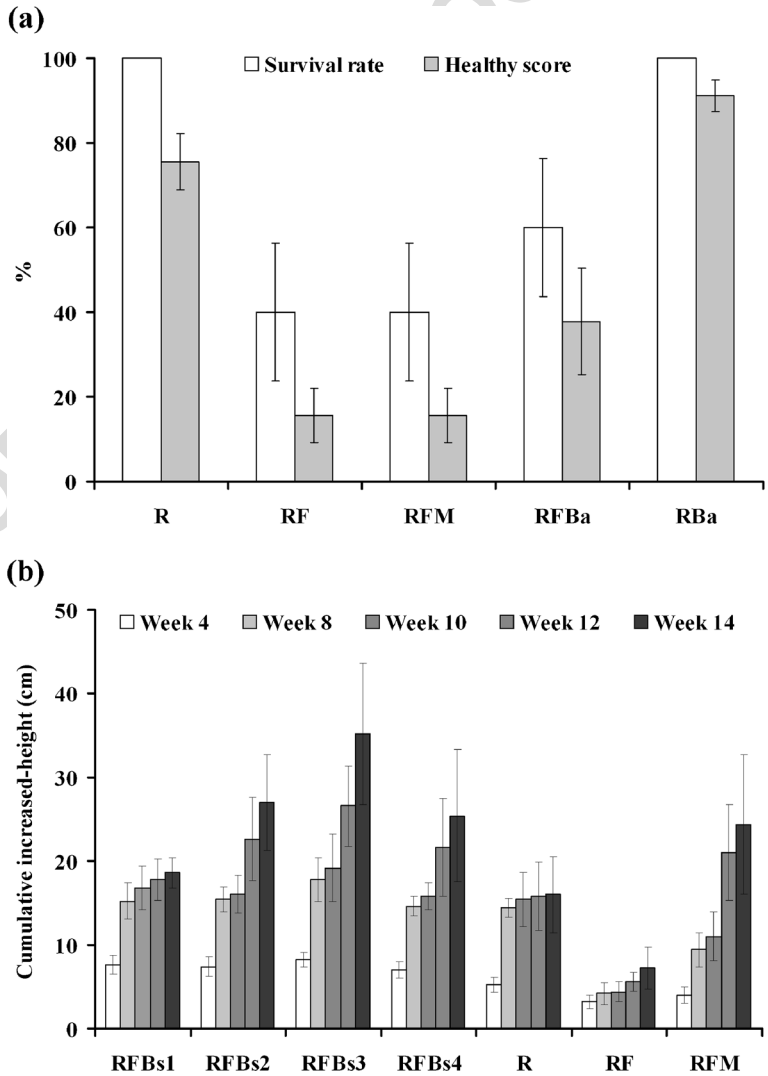

Fig. 6 Applications of biocontrol agent (Ba) and biocontrol starter (Bs) for suppression of white root disease caused by *Rigidoporus* sp. in nursery stage of rubber tree farming. Survival rate and healthy score of rubber trees growing in agricultural soil (R), were compared to other treatments (see also Materials and Methods) (**a**). Ten rubber trees were used in each treatment (**a**). Means of cumulative increased-height of rubber trees growing in each treatment mentioned previously together with the application of biocontrol starters (Bs1-4) were compared (see also Materials and Methods for the composition of each starter) (**b**). Five rubber trees were used in each treatment (**b**)

Discussion

Our results indicate that the genus *Streptomyces* is one of the predominant bacteria found in soil habitats. Its number isolated from the rhizospheric soils was higher than other actinobacterial genera. The different level of actinobacterial abundance might due to either the variations of physicochemical and biological properties of the rhizospheric soils or the selective association between plant and microorganisms (Jayasinghe and Parkinson 2007; Pandey and Palni 2007; Tewtrakul and Subhadhirasakul 2007). Mostly, *Streptomyces* is a representative producer of diverse bioactive compounds that are used in various disciplines. Although the potent antifungal isolate TM32 was phylogenetically related to *Streptomyces sioyaensis*, it showed some dissimilar phenotypes to its closest species. It was found that the isolate possesses chitinase activity but no β-1,3-glucanase activity. Otherwise, β-1,3-glucanase was reported previously as a key tool of *S. sioyaensis* for its antifungal activity (Hong et al. 2002, 2008). We supposed that the isolate TM32 might be a novel candidate of the genus *Streptomyces*, while its complete taxonomic evaluation using polyphasic approaches would be further required to support its novelty.

Abilities to produce cellulolytic enzymes and antifungal activity were observed in all distinct antifungal actinobacteria. The isolate GN15 could produce various enzymes but showed lowest inhibitory level against the fungus. On the other hand, a small amount of CCB obtained from the isolate TM32 could inhibit the growth of the fungus equal to a gram of metalaxyl. Although it was the most potent antifungal actinobacterium, it was able to produce only chitinase as a cellulolytic enzyme. This might due to either the cell wall structure of the pathogenic fungus, comprising chitin, or different modes of action constituted by the antifungal isolate. Yu et al. (2008) mentioned that chitinolytic microorganisms are considered to be more effective antagonists of fungal pathogens because of the direct action of chitinase alone. Based on our observation, the antifungal activity of the isolate TM32 might not only due to the action of chitinase but also the mixture of diverse bioactive compounds that remain in its crude culture fluid such as antibiotics, antifungal agents, siderophore, etc.

Application of *Streptomyces* sp. TM32 as a biocontrol agent at the nursery stage of rubber tree cultivation did not harm to the trees, and could suppress the disease caused by the fungus. In general, rhizospheric actinobacteria are not only a defence against soil borne pathogens but they can also colonize on plant root and promote the plant growth. The capacity to suppress the disease in nursery farming of rubber trees by our biocontrol agents showed similar results to chemical treatment. However, it was found that the suppression potential was greater when the biocontrol starters were applied. Uses of both chemical and biocontrol agent treatment seemed to be influenced by technical dilution through watering process. This process may limit the plant root colonization of the rhizospheric actinobacteria, while the solid state cultivation of them in the form of biocontrol starters could allow persistent growth of their mycelia and later promote their mutual coexistence in the rhizospheric habitat.

Widespread use of synthetic chemicals to control various fungal pathogens in agriculture can induce resistance to those chemicals (Para and Ristaino 1998). With the aim to reduce chemical use in agriculture, biological control could be an alternative approach for the farmers. Utilization of plant-microbe association might be a sustainable method of control of plant pathogens. Here, we conclude that use of biocontrol starters from *Streptomyces* sp. TM32 could suppress the white root disease caused by *Rigidoporus* sp. in nursery farming of rubber trees. It could also promote and immunize the health of rubber tree seedlings, which would be a great start for further long-term plantation of rubber trees in the farm-scale.

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