

Metadata of the article that will be visualized in OnlineFirst

1	Article Title	Utilization of rhizospheric <i>Streptomyces</i> for biological control of <i>Rigidoporus</i> sp. causing white root disease in rubber tree
2	Article Sub- Title	
3	Article Copyright - Year	Koninklijke Nederlandse Planteziektenkundige Vereniging 2015 (This will be the copyright line in the final PDF)
4	Journal Name	European Journal of Plant Pathology
5		Family Name Nakaew
6		Particle
7		Given Name Nareeluk
8		Suffix
9		Organization Naresuan University
10	Corresponding Author	Division Department of Microbiology and Parasitology, Faculty of Medical Science
11		Address Phitsanulok 65000, Thailand
12		Organization Naresuan University
13		Division Centre of Excellent in Fungal Research, Faculty of Medical Science
14		Address Phitsanulok 65000, Thailand
15		e-mail nnakaew@hotmail.com
16		Family Name Rangjaroen
17		Particle
18		Given Name Chakrapong
19		Suffix
20	Author	Organization Phranakhon Rajabhat University
21		Division Department of Agricultural Management Technology, Faculty of Science and Technology
22		Address Bangkhen 10220, Bangkok, Thailand
23		e-mail
24		Family Name Sungthong
25	Author	Particle
26		Given Name Rungroch
27		Suffix

28	Organization	Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS-CSIC)
29	Division	
30	Address	Apartado 1052, Seville 41080, Spain
31	e-mail	
32	Received	
33	Schedule Revised	
34	Accepted	7 January 2015
35	Abstract	<p>White root disease causing by <i>Rigidoporus</i> 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 (ED₅₀) 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 <i>Streptomyces sioyaensis</i> supported by 99 % similarity of 16S rRNA gene sequences. We concluded that application of the biocontrol starters produced by this <i>Streptomyces</i> isolate would be an alternative approach for sustainable control of soil-borne fungal invasion in long-term rubber tree farming.</p>
36	Keywords separated by ' - '	<i>Hevea brasiliensis</i> - Soil-borne fungal pathogen - Biocontrol - Actinobacteria - Antifungal activity
37	Foot note information	

Utilization of rhizospheric *Streptomyces* for biological control of *Rigidoporus* sp. causing white root disease in rubber tree

Nareeluk Nakaew · Chakrapong Rangjaroen ·
Rungroch Sungthong

Accepted: 7 January 2015
© Koninklijke Nederlandse Planteziektenkundige Vereniging 2015

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 (ED₅₀) 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 siوياensis* 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

Introduction

Rubber trees, known also as para rubber trees (*Hevea 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.

N. Nakaew (✉)
Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Phitsanulok 65000, Thailand
e-mail: nnakaew@hotmail.com

N. Nakaew
Centre of Excellent in Fungal Research, Faculty of Medical Science, Naresuan University, Phitsanulok 65000, Thailand

C. Rangjaroen
Department of Agricultural Management Technology, Faculty of Science and Technology, Phranakhon Rajabhat University, Bangkok, Bangkok 10220, Thailand

R. Sungthong
Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS-CSIC), Apartado 1052, Seville 41080, Spain

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 Soyong 2010), *Lentinus squarrosulus* (Idwan et al. 1992), *Chaetomium* spp. and *Aspergillus niger* (Kaewchai and Soyong 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

152	after growing the fungus on 100 g of sterilized wheat	phenotypic data (morphological characteristics of	197
153	grains at 30 °C for 15 days, and the whole fungal culture	mycelia and spores, and cell wall diaminopimelic acid	198
154	was inoculated at the bottom of a pot before planting	(DAP)) into three groups including <i>Streptomyces</i> ,	199
155	rubber trees. Budded rubber trees (cultivar RRIM600) at	<i>Micromonospora</i> and unknown genus. The	200
156	an age of 8–10 months were purchased from Wangthong	actinobacterial isolates were grown on International	201
157	district, Phitsanulok, Thailand and used throughout this	<i>Streptomyces</i> project medium II (ISP2) agar (Atlas	202
158	work. The fungus was also isolated from infected roots	1946) at 30 °C for a number of days, allowing them to	203
159	of diseased rubber trees grown at the Rubber Research	form mature spores. Morphological observations were	204
160	Center. Briefly, the infected roots were washed by run-	carried out microscopically under a light microscope	205
161	ning tap water to remove soil particles for 10 min and	(Olympus BH-2, Japan). DAP isomeric analysis was	206
162	washed twice with sterilized distilled water. Small	done following a procedure described by Hasegawa	207
163	pieces of the roots were cut and put on Potato dextrose	et al. (1983).	208
164	agar (PDA) (Difco™, MD, USA) plus chloramphenicol		
165	and streptomycin at the final concentration of 50 mg l ⁻¹	Screening of antifungal actinobacteria	209
166	each. The agar plates were then incubated at 25 °C until		
167	the fungal mycelia were growing out of the roots. Iso-	Antifungal activity of all rhizospheric actinobacteria	210
168	lated fungi were sub-cultured until they were pure cul-	was assayed against <i>Rigidoporus</i> sp. by dual culture	211
169	tures and identified preliminarily based on morpholog-	technique. Each actinobacterial isolate was grown by	212
170	ical observations compared to known <i>Rigidoporus</i> sp.	streak-plate method on ISP2 agar at 30 °C for 7 days,	213
171	The observations were done microscopically and mac-	where an agar plug (5 mm Ø) of the fungus grown	214
172	roscopically. All isolates of <i>Rigidoporus</i> were allowed	previously on PDA at 30 °C for 7 days was placed	215
173	to grow on PDA and maintained at 4 °C for further use.	4 cm from the actinobacterial streak line. The dual	216
		culture plate was incubated at 30 °C, where the antifun-	217
174	Isolation of actinobacteria from rhizospheric soils	gal activity was observed everyday. Size of any apparent	218
		inhibition zone at the interaction area of both microor-	219
175	Rhizospheric soils were collected from the field of medi-	ganisms was measured (approximately a week after	220
176	cinical plants: turmeric (<i>Curcuma longa</i>) and ginger	incubation). Antagonistic effects of the actinobacteria	221
177	(<i>Zingiber officinale</i>) in Chiang Mai province, Thailand.	on the physiology of the fungal mycelia at the inhibition	222
178	The soils were air-dried at ambient temperature prior to	zone were observed microscopically. Different antifun-	223
179	oven-drying at 120 °C for 1 h (Tamura et al. 1997).	gal levels were determined by different sizes of the	224
180	Dried soil samples were then pretreated with 1.5 % (w/v)	inhibition zones (Table 1).	225
181	phenol solution (Hayakawa et al. 2004). The superna-		
182	tant of this solution was serially diluted by sterilized	Phylogenetic analysis of the most potent actinobacterial	226
183	distilled water before spreading on Humic acid-vitamin	isolate	227
184	agar (Hayakawa and Nonomura 1987) plus nystatin and		
185	cycloheximide at the final concentration of 25 and	The most potent actinobacterial isolate that exhibited the	228
186	10 µg ml ⁻¹ , respectively. The agar plates were incubated	largest inhibition zone against <i>Rigidoporus</i> sp. was se-	229
187	at 30 °C for a month. All visible actinobacterial isolates	lected. Its genomic DNA was extracted following the	230
188	were selected, sub-cultured and purified individually on	method described by Hopwood et al. (1985). The 16S	231
189	Hickey-Tresner (HT) agar (Atlas 1946), which were	rRNA gene was amplified by polymerase chain reaction	232
190	further maintained at 4 °C as a working stock and in	(PCR) using a pair of universal primers: 27 F (5'-AGAG	233
191	20 % (v/v) glycerol at -20 °C for a long term storage.	TTTGATCMTGGCTCAG-3') and 1525R (5'-AAGG	234
		AGGTGWTCCARCC-3') (Lane 1991). PCR product	235
192	Determination of generic abundance of rhizospheric	was purified using QIAquick PCR Purification Kit	236
193	actinobacteria	(Qiagen) and sequenced by Macrogen Inc. (Seoul, Ko-	237
		rea). The gene sequence obtained was compared with all	238
194	Total actinobacterial isolates obtained from the same	accessible sequences in GenBank database using	239
195	amount of each rhizospheric soil were counted. These	BLASTN software. Multiple sequence alignments and	240
196	rhizospheric actinobacteria were classified using some	phylogenetic tree were carried out using the neighbour-	241

Table 1 Actinobacteria isolated from rhizospheric soils of medicinal plants

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

242 joining method (Saitou and Nei 1987) in the Molecular
243 Evolutionary Genetics Analysis (MEGA) program ver-
244 sion 4 (Tamura et al. 2007), where the bootstrap confi-
245 dence levels were computed based on 1000 replications.

246 Evaluation of in vitro antifungal activity

247 Some antifungal actinobacteria (giving relatively large
248 (≥ 16 mm) inhibition zones) were evaluated in their
249 modes of antifungal action including productions of
250 antifungal agents, siderophores and cell wall degrading
251 enzymes. Briefly, the antifungal actinobacteria were
252 grown individually in 100 ml of ISP2 broth at 30 °C
253 with shaking at 120 rpm for 7 days, while their cell-free
254 culture broths (CCBs) were prepared by filtering
255 through 0.20- μm filter paper (Millipore Corporation,
256 Benford, MA). Antifungal activity of the CCBs against
257 *Rigidoporus* sp. was confirmed by agar well diffusion
258 assay. Inhibition capacity (% inhibition) of the CCBs
259 was evaluated using dry weight of fungal biomass (dry-
260 ing at 55 °C for 3 days) after growth at 30 °C for 20 days
261 on PDA supplemented with each CCB (at variant con-
262 centrations of 10, 20, 30, 40 and 50 % (v/v)) that was
263 covered with cellophane. The dry weight of fungal
264 biomass after growth in the same conditions on PDA
265 without CCB supplement was used as a control. The
266 CCB giving the highest % inhibition was evaluated by
267 its median effective dose (ED₅₀) for inhibition of fungal
268 growth compared with known concentrations of a chemi-
269 cal fungicide, Metalaxyl (Local trademark, Samut
270 Prakan, Thailand).

271 The production of siderophores was assayed using
272 Chrome azurol S (CAS) agar (Schwyn and Neilands
273 1987). The CCBs were individually applied to both

274 iron-restricted and iron-rich CAS agars. Appearance of
275 a yellow to orange halo on the blue CAS agar according
276 to the production of siderophores was observed, where
277 the diameter of the halo was measured.

278 Production of some cell wall degrading enzymes
279 (β -1,3-glucanase, cellulase, chitinase and protease)
280 was assayed by growing the antifungal
281 actinobacteria at 30 °C for 7 days on a set of agar
282 media supplemented with respective substrates of
283 the enzymes tested. The agar media used were
284 Minimal agar (% (w/v) of yeast extract, 0.2;
285 KH₂PO₄, 0.1; MgSO₄, 0.5; agar powder, 1.5) plus
286 0.5 % (w/v) of carboxymethyl cellulose (CMC) for
287 glucanolytic and cellulolytic activities, Chitin agar
288 (Malviya et al. 2009) for chitinolytic activity, and
289 Skim milk agar (Atlas 1946) for proteolytic activ-
290 ity. Staining with an aqueous solution of
291 1 mg ml⁻¹ Congo red for 15 min followed by
292 washing with 1 M NaCl solution was applied for
293 observations of glucanolytic and cellulolytic activ-
294 ities (Teather and Wood 1982). The enzyme activ-
295 ities of CCBs derived from the antifungal
296 actinobacteria were also determined, while these
297 CCBs were prepared after the actinobacterial
298 growth at 30 °C by shaking at 120 rpm, for 7 days
299 in 100 ml of the liquid media mentioned previous-
300 ly, except for the chitinase activity that Chitin-
301 peptone medium (pH 6.8) (Malviya et al. 2009)
302 was used instead. The β -1,3-glucanase and cellu-
303 lase activities of the CCBs were measured by
304 dinitrosalicylic acid method (DNS) Cattelan et al.
305 (1999), while the chitinase activity was measured
306 following the protocol described by Malviya et al.
307 (2009). The protease activity was measured

- 308 according to the protocol described by Yang and
309 Huang (1994).
- 310 Evaluation of plant growth promoting potentials
- 311 Some plant growth promoting potentials of the antifun-
312 gal actinobacteria such as productions of indole-3-acetic
313 acid (IAA) and ammonia together with capable of solu-
314 bilization of phosphate, were assessed. The ability to
315 produce IAA was evaluated following the method de-
316 scribed by Bano and Musarrat (2003). Briefly, one ml of
317 the same CCB as used for antifungal activity test was
318 mixed with 2 ml of Salkowski's reagent, where the
319 appearance of a pink colour indicates IAA production.
320 Optical density at 530 nm was used to estimate the level
321 of IAA produced compared with an IAA standard curve.
- 322 A full loop of each isolate of the actinobacteria was
323 inoculated into 10 ml Peptone water (Atlas 1946) and
324 incubated at 30 °C for 48 to 72 h. Nessler's reagent
325 (0.5 ml) was added into the culture broth, where the
326 colour brown turning to yellow was determined as posi-
327 tive ammonia production.
- 328 The ability of the actinobacteria to solubilize inor-
329 ganic phosphate was assessed using Pikovskaya's agar
330 (pH 7) (Nautiyal 1999). The actinobacteria were inocu-
331 lated on the agar by spot technique, and incubated at
332 30 °C for 7 days. Appearance of a clear zone reflecting
333 the removal of phosphate was determined as a positive
334 phosphate solubilization. Size of the clear zone was
335 measured by subtracting the diameter of the
336 actinobacterial colony.
- 337 Suppression of white root disease in nursery stage
338 of rubber trees
- 339 The budded rubber trees, cultivar RRIM600 as men-
340 tioned previously, were screened based on similarities of
341 fertility and size. Agricultural loamy soil was used nat-
342 urally for planting the rubber trees in all treatments. Five
343 treatments were carried out: 1) a control of planted
344 rubber trees untreated (R), 2) a pathogenic treatment of
345 R invaded by the fungus (RF), 3) a chemical treatment
346 of RF with metalaxyl (RFM), 4) a supplemental treat-
347 ment of R with the biocontrol agents (RBa), and 5) a
348 treatment of RF with the biocontrol agents (RFBa). The
349 soil invaded with the fungus was prepared by inoculat-
350 ing the whole volume of *Rigidoporus* sp. growing on
351 100 g of sterilized wheat grains at 30 °C for 15 days,
352 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 day-
light 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, yel-
lowish 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, in-
cluding: 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 condi-
tion. The sterilized materials (160 cm³) 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 con-
centration 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 rub-
ber 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

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

all areas of fibrous and lateral roots of the trees. 412
The macroscale observation of the disease symp- 413
toms is shown in Fig. 1. 414

402 Results

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

405 *Rigidoporus* sp. caused the disease in nursery rubber
406 trees within week 6–8 after it was introduced into
407 the soil for planting the rubber trees. Symptoms of
408 the disease began with the occurrence of yellowish
409 leaves (week 7) followed by burned leaves (week
410 10). Infected nursery rubber trees died at week
411 12–13, while the rhizomorph of the fungus covers

Actinobacteria from rhizospheric soils of medicinal 415
plants 416

A total of actinobacteria (209 isolates) was isolated 417
from rhizospheric soils of medicinal plants 418
(Table 1). Most of the actinobacteria (61 %) were 419
found in the rhizospheric soils of ginger. On the 420
basis of preliminary phenotypic classification, 421
Streptomyces was the dominant group of 422
actinobacteria found in rhizospheric soils of both 423
ginger (65 %) and turmeric (58 %). Most of the 424
actinobacteria (78 %) were inactive against 425

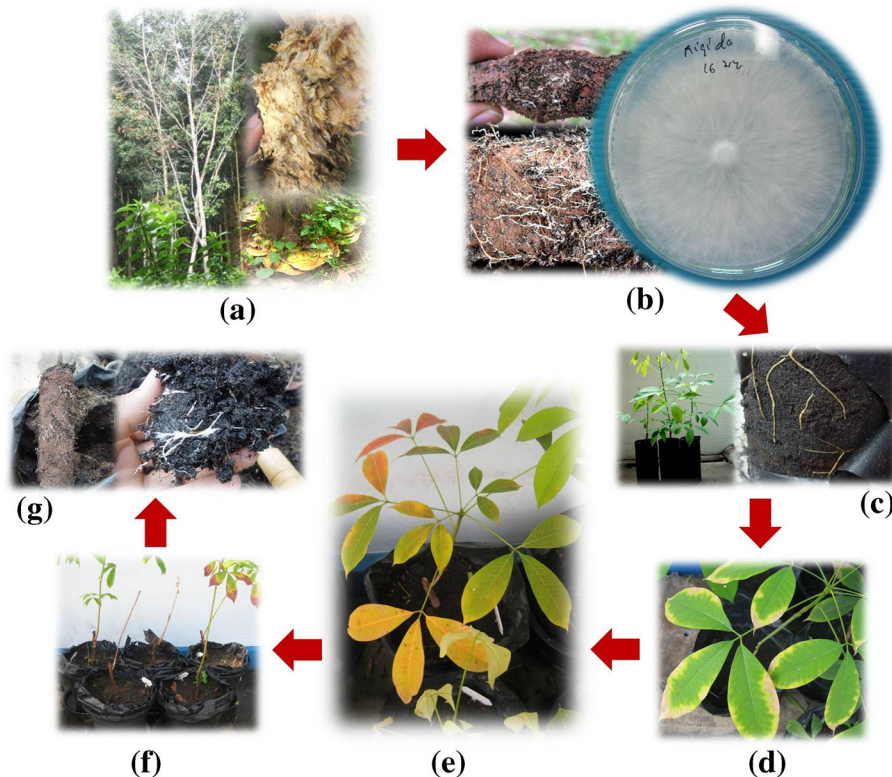


Fig. 1 The macroscale observation of the white root disease caused by *Rigidoporus* sp. Dead rubber tree after infection by the fungus for several months standing in farm without leaf (**a-left**), while the fungus formed fruit body at the base of the rubber tree (**a-right down**) where the white fungal 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

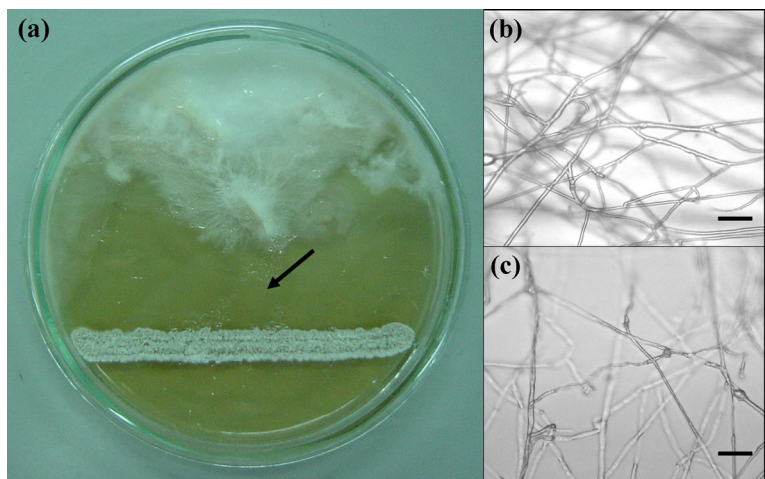
t2.2	Modes of action and plant growth promoting potentials	Actinobacterial isolate			
t2.3		TM32	GN12	GN15	GN20
t2.4	Antagonistic activity [inhibition zone (mm)]	24.00±1.00	18.33±0.58	16.00±1.00	16.67±0.58
t2.5	Siderophore production [halo zone (mm)]	11.75±0.96	8.00±1.41	18.50±1.29	5.25±0.50
t2.6	β-1,3-glucanase	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

426 *Rigidoporus* sp., while four actinobacterial isolates
 427 (TM32, GN12, GN15 and GN20) showed distinct
 428 antifungal activity by giving relatively large inhi-
 429 bition zone (≥16 mm). Only two (TM32 and
 430 GN12) out of these four isolates belonged to the
 431 *Streptomyces* group. Isolate TM32 originating from
 432 the rhizospheric soil of turmeric was determined as
 433 the most potent actinobacterial isolate, giving the
 434 largest inhibition zone (24.00±1.00 mm) against
 435 the fungus (Table 2). The antifungal activity of
 436 this isolate at the inhibition zone revealed that

437 the isolates produced antifungal substances causing
 438 the fungal mycelial wilt (Fig. 2). The isolate was
 439 classified into the genus *Streptomyces* on the basis
 440 of its morphology to form long spiral chain spores
 441 (Fig. 3) and its cell wall comprised of LL-DAP
 442 and glycine, which is the cell wall chemotype I of the
 443 genus *Streptomyces* (Lechevalier and Lechevalier
 444 1970). In addition, a total 1452 nucleotides sequence
 445 of its 16S rRNA gene revealed it was closely related to
 446 *Streptomyces sioyaensis* (DQ026654) supported by
 447 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



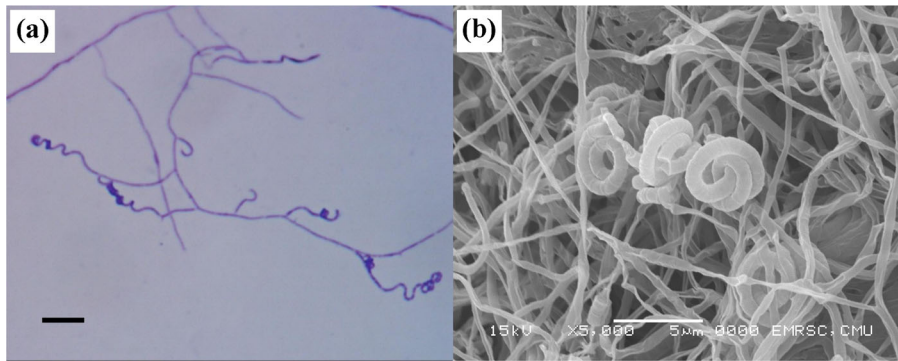


Fig. 3 Morphology of *Streptomyces* sp. TM32. Isolate TM32 was grown on HT agar at 30 °C for 10 days. Then, the aerial mycelia and spores of isolate TM32 were observed and photographed under light (a) and scanning electron (b) microscopes. Bars=5 µm

448 Antifungal activity and plant growth promoting
 449 potentials of the rhizospheric actinobacteria

450 Modes of action and some plant growth promoting
 451 potentials of the four distinct actinobacterial isolates
 452 are shown in Table 2. They inhibited the growth of

Rigidoporus sp. with different antifungal levels deter- 453
 mined by the sizes of inhibition zones. They could also 454
 produce siderophores and at least one cellulolytic en- 455
 zyme, but no production of protease was observed. 456
 Isolate TM32 showed the highest chitinase activity 457
 (0.093±0.004 U mg⁻¹) followed by isolates GN15 and 458

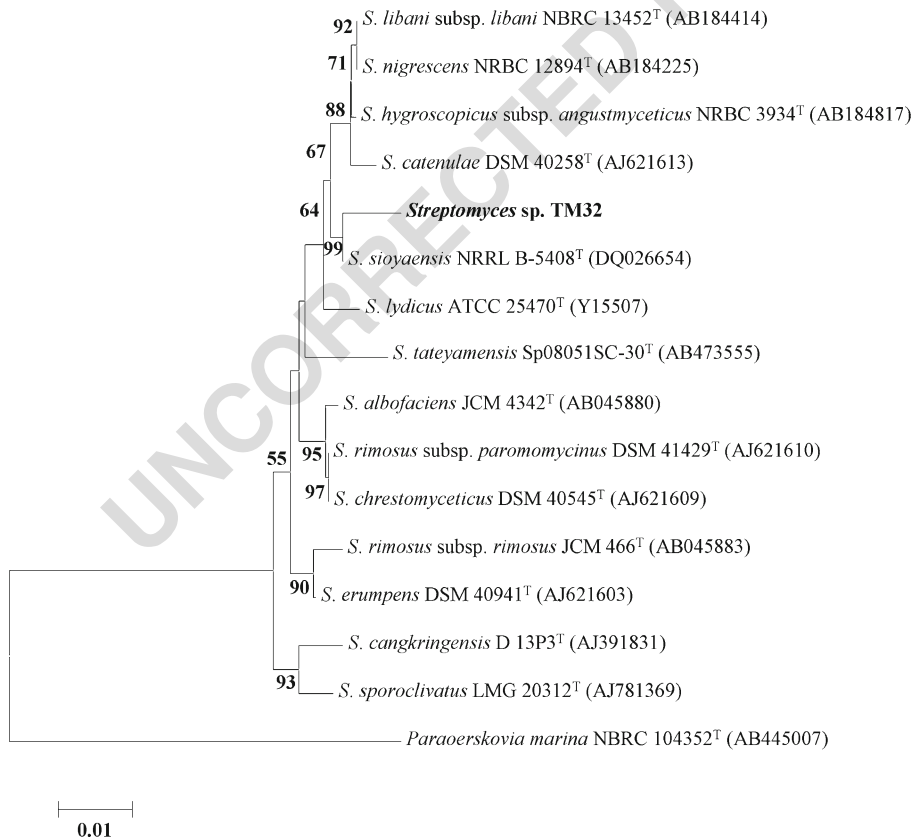


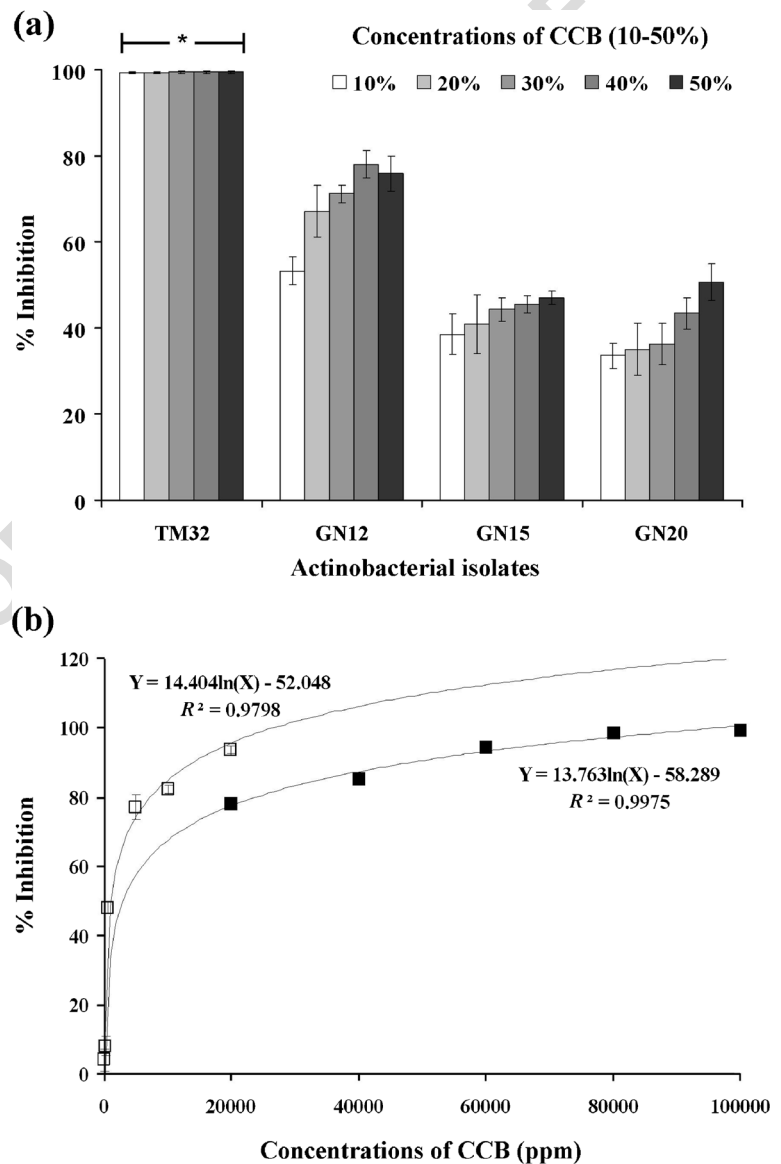
Fig. 4 Phylogenetic tree based on the 16S rRNA gene sequence of *Streptomyces* sp. TM32. The gene sequence (1452 nucleotides without gap) of isolate TM32 (in bold) was aligned and compared to the available sequences in GenBank database (accession

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}*

459 GN20. In vitro antifungal activities of the CCBs
 460 derived from the four isolates are shown in
 461 Fig. 5a. The CCB derived from isolate TM32
 462 could inhibit the fungal growth nearly 100 %,
 463 while its inhibition values were not different sig-
 464 nificantly across the different concentrations tested
 465 ($F_{(4, 15)}=0.62$, $P=0.0001$). At the same concentra-
 466 tion (10^{F1} , 20^{F2} , 30^{F3} , 40^{F4} and $50\%^{F5}$ (v/v))
 467 compared, the CCB of isolate TM32 showed sig-
 468 nificantly greater % inhibition than those of other
 469 CCBs derived from other isolates ($F_{1-5(3, 12)}=$
 470 346.92, 117.29, 361.88, 426.98 and 256.16, $P=$

0.0001). Median effective dose (ED_{50}) of this
 471 CCB calculated by logistic regression equation
 472 was 2.61 ml l^{-1} or 0.26% (v/v) compared with a
 473 standard curve of the known concentrations of
 474 metalaxyl (Fig 5b). Some plant growth promoting
 475 potentials of the four isolates are shown in Table 2.
 476 All isolates could produce IAA, while the highest pro-
 477 duction was found by isolate GN20 ($72.50 \pm$
 478 $2.18 \mu\text{g ml}^{-1}$). The isolate GN20 was also the greatest
 479 phosphate solubilizer, giving the largest solubilizing
 480 zone ($15.17 \pm 0.76 \text{ mm}$). Only the isolates GN15 and
 481 GN20 were able to produce ammonia.
 482

Fig. 5 In vitro antifungal activity of actinobacteria isolated from rhizosphere soils of turmeric (TM) and ginger (GN). Percent inhibition (mean \pm 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

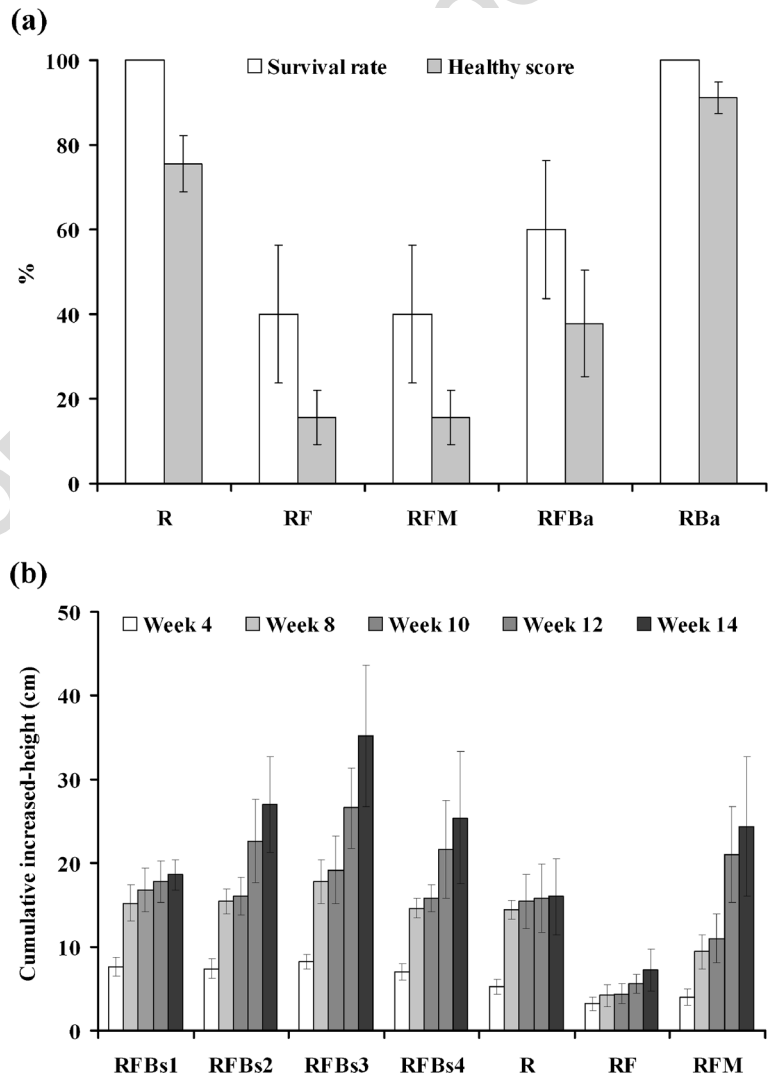


483 Utilization of *Streptomyces* sp. TM32 for suppression
484 of white root disease in rubber trees

485 Uses of both biocontrol agents and biocontrol starters in
486 nursery stage could suppress the disease causing by
487 *Rigidoporus* sp. (Fig. 6). The highest survival rate
488 (100 %) and healthy score (91 %) of rubber trees were
489 observed in the supplemental treatment (RBa) (Fig. 6a).
490 The chemical treatment (RFM) showed equal both sur-
491 vival rate (40 %) and healthy score (16 %) to the
492 pathogenic treatment (RF). The biocontrol agents could
493 suppress the disease caused by the fungus (RFBa) to
494 approximately 20 % greater extent than the chemical
495 treatment. Modification of biocontrol agents in the
496 forms of biocontrol starters (Bs1-4) was carried out

497 using different holding materials for sustaining the
498 growth of isolate TM32. No dead rubber tree was ob-
499 served when any biocontrol starters were applied. Potent-
500 ial of each biocontrol starter was determined by cumu-
501 lative increased height of the rubber trees grown in fungal
502 invaded soil (RF) treated with each biocontrol starter
503 (Fig. 6b). Among all biocontrol starters, starter Bs3 pre-
504 pared with sorghum grain was the optimal one for sup-
505 pression of the disease, revealing the highest cumulative
506 increased-height at week 14 of the experiments. Howev-
507 er, the statistical comparison demonstrated that the po-
508 tential of this starter was not significantly different from
509 starter Bs2, starter Bs4 and chemical treatment but sig-
510 nificantly different from starter Bs1, the control and the
511 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)



512 **Discussion**

513 Our results indicate that the genus *Streptomyces* is one
 514 of the predominant bacteria found in soil habitats. Its
 515 number isolated from the rhizospheric soils was higher
 516 than other actinobacterial genera. The different level of
 517 actinobacterial abundance might due to either the vari-
 518 ations of physicochemical and biological properties of
 519 the rhizospheric soils or the selective association be-
 520 tween plant and microorganisms (Jayasinghe and
 521 Parkinson 2007; Pandey and Palni 2007; Tewtrakul
 522 and Subhadhirasakul 2007). Mostly, *Streptomyces* is a
 523 representative producer of diverse bioactive compounds
 524 that are used in various disciplines. Although the potent
 525 antifungal isolate TM32 was phylogenetically related to
 526 *Streptomyces siوياensis*, it showed some dissimilar phe-
 527 notypes to its closest species. It was found that the
 528 isolate possesses chitinase activity but no β -1,3-
 529 glucanase activity. Otherwise, β -1,3-glucanase was re-
 530 ported previously as a key tool of *S. siوياensis* for its
 531 antifungal activity (Hong et al. 2002, 2008). We sup-
 532 posed that the isolate TM32 might be a novel candidate
 533 of the genus *Streptomyces*, while its complete taxonom-
 534 ic evaluation using polyphasic approaches would be
 535 further required to support its novelty.

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

557 Application of *Streptomyces* sp. TM32 as a biocon-
 558 trol agent at the nursery stage of rubber tree cultivation
 559 did not harm to the trees, and could suppress the disease

caused by the fungus. In general, rhizospheric 560
 actinobacteria are not only a defence against soil borne 561
 pathogens but they can also colonize on plant root and 562
 promote the plant growth. The capacity to suppress the 563
 disease in nursery farming of rubber trees by our bio- 564
 control agents showed similar results to chemical treat- 565
 ment. However, it was found that the suppression po- 566
 tential was greater when the biocontrol starters were 567
 applied. Uses of both chemical and biocontrol agent 568
 treatment seemed to be influenced by technical dilution 569
 through watering process. This process may limit the 570
 plant root colonization of the rhizospheric 571
 actinobacteria, while the solid state cultivation of them 572
 in the form of biocontrol starters could allow persistent 573
 growth of their mycelia and later promote their mutual 574
 coexistence in the rhizospheric habitat. 575

576 Widespread use of synthetic chemicals to control 577
 various fungal pathogens in agriculture can induce re- 578
 sistance to those chemicals (Para and Ristaino 1998). 579
 With the aim to reduce chemical use in agriculture, 580
 biological control could be an alternative approach for 581
 the farmers. Utilization of plant-microbe association 582
 might be a sustainable method of control of plant path- 583
 ogens. Here, we conclude that use of biocontrol starters 584
 from *Streptomyces* sp. TM32 could suppress the white 585
 root disease caused by *Rigidoporus* sp. in nursery farm- 586
 ing of rubber trees. It could also promote and immunize 587
 the health of rubber tree seedlings, which would be a 588
 great start for further long-term plantation of rubber 589
 trees in the farm-scale. 590

Acknowledgment This research was fully funded by the Thai- 591
 land Research Fund (TRF) under the Small Projects on Rubber 592
 (SPR) with a grant no. RDG5250036 and RDG5350024. We 593
 thank Prof. Dr. Gavin Reynolds, Biomedical Science Research 594
 centre, Sheffield Hallam University, UK for his kind assistance 595
 on improvement of English usage. 596
 597

References

- Atlas, R. M. (1946). *Handbook of microbiological media* (2nd 600
 ed.). Florida: CRC Press. 601
 Bano, N., & Musarrat, J. (2003). Characterization of a new 602
Pseudomonas aeruginosa strain NJ-15 as a potential biocon- 603
 trol agent. *Current Microbiology*, 46, 324–328. 604
 Business Report Thailand. (2011). Thai Rubber Industry Success. 605
 Business Report Thailand, Issue 6. [http://www. 606
 businessreportthailand.com/thai-rubber-industry-success- 607
 12632](http://www.businessreportthailand.com/thai-rubber-industry-success-12632)). Accessed 8 Dec 2011. 608

- 609 Cattelan, A. J., Hartel, P. G., & Fuhrmann, J. J. (1999). Screening
610 for plant growth-promoting rhizobacteria to promote early
611 soybean growth. *Soil Science Society of American Journal*,
612 63, 1670–1680.
- 613 Evueh, G. A., & Ogbemor, N. O. (2008). Use of phylloplane fungi
614 as biocontrol agent against *Colletotrichum* leaf disease of
615 rubber (*Hevea brasiliensis* Muell. Arg.). *African Journal of*
616 *Biotechnology*, 7, 2569–2572.
- 617 Evueh, A. G., Okhuoya, J. A., Osemwegie, O. O., Attitalla, I. H.,
618 & Ogbemor, O. N. (2011). Evaluation of phylloplane fungi as
619 biocontrol agent of *Corynespora* leaf fall disease of rubber
620 (*Hevea brasiliensis* Muell. Arg.). *World Journal of Fungal*
621 *and Plant Biology*, 2, 01–05.
- 622 Hasegawa, T., Takizawa, M., & Tanida, S. (1983). A rapid analysis
623 for chemical grouping of aerobic actinomycetes. *Journal of*
624 *General and Applied Microbiology*, 29, 319–322.
- 625 Hayakawa, M., & Nonomura, H. (1987). Humic acid-vitamin
626 agar, a new medium for the selective isolation of soil actino-
627 mycetes. *Journal of Fermentation Technology*, 65, 501–509.
- 628 Hayakawa, M., Yoshida, Y., & Iimura, Y. (2004). Selective isola-
629 tion of bioactive soil actinomycetes belonging to the
630 *Streptomyces violaceusniger* phenotypic cluster. *Journal of*
631 *Applied Microbiology*, 96, 973–981.
- 632 Hong, T. Y., Cheng, C. W., Huang, J. W., & Meng, M. (2002).
633 Isolation and biochemical characterization of an endo-1,3- β -
634 glucanase from *Streptomyces sioyaensis* containing a C-
635 terminal family 6 carbohydrate-binding module that binds
636 to 1,3- β -glucan. *Microbiology*, 148, 1151–1159.
- 637 Hong, T. Y., Hsiao, Y. Y., Meng, M., & Li, T. T. (2008). The 1.5 Å
638 structure of endo-1,3- β -glucanase from *Streptomyces*
639 *sioyaensis*: evolution of the active-site structure for 1,3- β -
640 glucan-binding specificity and hydrolysis. *Acta*
641 *Crystallographica Section D Biological Crystallography*,
642 64, 964–970.
- 643 Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Bruton, C.
644 J., Kieser, H. M., Lydiate, D. J., Smith, C. P., Ward, J. M., &
645 Schrempf, H. (1985). Genetic Manipulation of *Streptomyces*.
646 A Laboratory Manual. John Innes Foundation Norwich, UK.
- 647 Idwan, S. L., Iraqi, H. A. I., Le Febvre, G., Kiffer, E., & Botton, B.
648 (1992). Screening of some basidiomycetes for biological
649 control of *Rigidoporus lignosus*, a parasite of rubber tree,
650 *Hevea brasiliensis*. *Mycological Research*, 96, 621–625.
- 651 Jayasinghe, B. A. T. D., & Parkinson, D. (2007). Actinomycetes as
652 antagonists of litter decomposer fungi. *Applied Soil Ecology*,
653 38, 109–118.
- 654 Jayasuriya, K. E., & Thennakoon, B. I. (2007). Biological control
655 of *Rigidoporus microporus*, the cause of white root disease in
656 rubber. *Ceylon Journal of Science (Biological Science)*, 36,
657 9–16.
- 658 Kaewchai, S., & Soyong, K. (2010). Application of biofungicides
659 against *Rigidoporus microporus* causing white root disease
660 of rubber trees. *Journal of Agricultural Science and*
661 *Technology*, 6, 349–363.
- 662 Kaewchai, S., Wang, H. K., Lin, F. C., Hyde, K. D., & Soyong, K.
663 (2009). Genetic variation among isolates of *Rigidoporus*
664 *microporus* causing white root disease of rubber trees in
665 Southern Thailand revealed by ISSR markers and pathogenic-
666 ity. *African Journal of Microbiology Research*, 3, 641–648.
- 667 Lane, D. J. (1991). 16S/23S rRNA sequencing. In E. Stackebrandt
668 & M. Goodfellow (Eds.), *Nucleic acid techniques in bacte-*
669 *rial systematics* (pp. 115–148). Chichester: Wiley.
- 670 Lechevalier, M. P., & Lechevalier, H. (1970). Chemical composi-
671 tion as a criterion in the classification of aerobic actinomy-
672 cetes. *International Journal of Systematic and Evolutionary*
673 *Microbiology*, 20, 435–443.
- 674 Loqman, S., Barka, E. A., Clement, C., & Ouhdouch, Y. (2009).
675 Antagonistic actinomycetes from Moroccan soil to control
676 the grapevine gray mold. *World Journal of Microbiology and*
677 *Biotechnology*, 25, 81–91.
- 678 Malviya, M. K., Pandey, A., Trivedi, P., Gupta, G., & Kumar, B.
679 (2009). Chitinolytic activity of cold tolerant antagonistic
680 species of *Streptomyces* isolated from glacial sites of Indian
681 Himalaya. *Current Microbiology*, 59, 502–508.
- 682 Nandris, D., Nicole, M., & Geiger, J. P. (1987a). Root rot diseases
683 of rubber trees. *Plant Disease*, 71, 298–306.
- 684 Nandris, D., Nicole, M., & Geiger, J. P. (1987b). Variation in
685 virulence among *Rigidoporus lignosus* and *Pheleinus noxius*
686 isolates from West Africa. *Sonderdruck aus European*
687 *Journal of Forest Pathology*, 17, 271–281.
- 688 Nautiyal, C. S. (1999). An efficient microbiological growth medi-
689 um for screening phosphate solubilizing microorganisms.
690 *FEMS Microbiology Letter*, 170, 265–270.
- 691 Nicole, M., & Benhamou, N. (1991). Cytochemical aspects of
692 cellulose breakdown during the infection process of rubber
693 tree roots infected by *Rigidoporus lignosus*. *Phytopathology*,
694 81, 1412–1420.
- 695 Ogbemor, N. O., Adekunle, A. T., Eghafona, N. O., & Ogboghodo,
696 A. I. (2010). *Ganoderma pseudoferreum*: Biological control
697 possibilities with microorganisms isolated from soils of rub-
698 ber plantations in Nigeria. *African Journal of General*
699 *Agriculture*, 6, 301–305.
- 700 Pandey, A., & Palni, L. M. S. (2007). The rhizosphere effect in
701 trees of the Indian Central Himalaya with special reference to
702 altitude. *Applied Ecology and Environmental Research*, 5,
703 93–102.
- 704 Para, G., & Ristaino, J. (1998). Insensitivity to Ridomil Gold
705 (mefenoxam) found among field isolates of *Phytophthora*
706 *capsici* causing *Phytophthora* blight on bell pepper in
707 North Carolina and New Jersey. *Plant Disease*, 82, 711–711.
- 708 Rose, K., & Steinbüchel, A. (2005). Biodegradation of natural
709 rubber and related compounds: recent insights into a hardly
710 understood catabolic capability of microorganisms. *Applied*
711 *and Environmental Microbiology*, 71, 2803–2812.
- 712 Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new
713 method for reconstructing phylogenetic trees. *Molecular*
714 *Biology and Evolution*, 4, 406–425.
- 715 Schwyn, B., & Neilands, J. B. (1987). Universal chemical assay
716 for the detection and determination of siderophores.
717 *Analytical Biochemistry*, 160, 47–56.
- 718 Tamura, T., Hayakawa, M., & Hatano, K. (1997). A new genus of
719 the order *Actinomycetales*, *Spirilliplanes* gen. nov., with de-
720 scription of *Spirilliplanes yamanashiensis* sp. nov.
721 *International Journal of Systematic Bacteriology*, 47,
722 97–102.
- 723 Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4:
724 Molecular Evolutionary Genetics Analysis (MEGA) soft-
725 ware version 4.0. *Molecular Biology and Evolution*, 24,
726 1596–1599.
- 727 Teather, R. M., & Wood, P. J. (1982). Use of Congo red polysac-
728 charide interactions complex formation between Congo red
729 and polysaccharide in detection and assay of polysaccharide
730 hydrolases. *Methods in Enzymology*, 160, 59–74.

- 731 Tewtrakul, S., & Subhadhirasakul, S. (2007). Anti-allergic activity
732 of some selected plants in the *Zingiberaceae* family. *Journal*
733 *of Ethnopharmacology*, *109*, 535–538. 742
- 734 Tokala, R. K., Strap, J. L., Jung, C. M., Crawford, D. L., Salove,
735 M. H., Deobald, L. A., Bailey, J. F., & Morra, M. J. (2002).
736 Novel plant-microbe rhizosphere interaction involving
737 *Streptomyces lydicus* WYEC108 and the pea plant (*Pisum*
738 *sativum*). *Applied and Environmental Microbiology*, *68*,
739 2161–2171. 743
- 740 Vasconcellos, R. L. F., & Cardoso, E. J. B. N. (2009).
741 Rhizospheric streptomycetes as potential biocontrol agents
742 of *Fusarium* and *Armillaria* pine rot and as PGPR for *Pinus*
743 *taeda*. *BioControl*, *54*, 807–816. 744
- 745 Wilhelm, S. (1973). Principles of biological control of soil-borne
746 plant diseases. *Soil Biology and Biochemistry*, *5*, 729–737. 745
- 747 Yang, S., & Huang, C. I. (1994). Protease production by amylo-
748 lytic fungi in solid state fermentation. *Journal of the Chinese*
749 *Agricultural Chemical Society*, *32*, 589–601. 746
- 750 Yu, T., Wang, L., Yin, Y., Wang, Y., & Zheng, X. (2008). Effect of
751 chitin on the antagonistic activity of *Cryptococcus laurentii*
752 against *Penicillium expansum* in pear fruit. *International*
753 *Journal of Food Microbiology*, *122*, 44–48. 751

UNCORRECTED PROOF

AUTHOR QUERY

AUTHOR PLEASE ANSWER QUERY.

No Query.

UNCORRECTED PROOF