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Screening of Antifungal Microorganisms with Strong Biological Activity against Oak Wilt Fungus, *Raffaelea quercus-mongolicae*

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Abstract

Since the mass mortality of *Quercus mongolica* has been first reported in Gyeonggi province at 2004, the disease spread rapidly over Korean peninsula annually. Ambrosia beetle (*Platypus koryoensis*) was known as the insect vector of oak wilt fungus, *Raffaelea quercus-mongolicae*, and control methods of the disease had mainly been focused on eradication of insect vector. However, for the efficient management of the disease, combined control methods for both of the pathogenic fungus and insect vector are strongly required. As one of the efforts to suppress the pathogenic fungus, antifungal activities of *Streptomyces* isolated from oak forest soil were assayed in this study. Optimum culture condition for the selected isolates was also studied, As a result, *Streptomyces blastmyceticus* cultured in PDB (Potato Dextrose Broth) at 25°C for 1 week showed the strongest antifungal activity against oak wilt fungus. Mycelial growth inhibition rates (MGIRs) of *Streptomyces* isolates were compared on culture media supplemented with heated and unheated culture filtrates of *S. blastmyceticus*. MGIRs on culture media with unheated culture filtrates were generally higher than those on culture media with heated culture filtrates. Antagonistic mechanism to get involved in the inhibition of hyphal growth and spore formation of the pathogen is due to the antifungal metabolites produced by *Streptomyces*. This study will provide the fundamental information in developing biocontrol agents for the environment-friendly management of oak wilt disease.

Key Words: screening of antifungal activity, oak wilt fungus, *Streptomyces blastmyceticus*, quercus mongolica, antifungal metabolites

Introduction

Biological control of plant pathogens by antagonistic or parasitic microorganisms has been studied for a long time in USA, Japan, China, India, Brazil, and European countries (Berdy 1974; Donadio et al. 2002; Lee 2015). Antagonistic microorganism include endophytic microorganisms, which inhibit mycelial growth, sporulation, and/or spore germination of plant pathogens by producing diffusable compounds (Osada 1998; Watve et al. 2001). On the other hand, mycoparasitic microorganisms destruct fungal cell walls physically by producing hydrolytic enzymes as biocontrol agents. However, most of these studies has been conducted for the control of perrenial host plants, but for woody plants efficient methods applicable in forest are not developed yet except several cases (Jeon et al. 2010;

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Lee et al. 2012; Lee et al. 2013; Lee et al. 2014; Lee et al. 2015)

Since oak wilt symptom was first noticed in oak forest in Korea at 2004 (Jung 2004), the affected area and infected trees were annually increased (Park et al. 2008). However, disease management was mainly focused on the control of an insect vector, *Platypus koryoensis* (Kuroda 2001; Kubono and Ito 2002). Cross section of the dead trees showed sapwood discoloration along the gallery made by the invasion of insect vector (Murata et al. 2007; Murata et al. 2009). This symptom was developed by colonization of disseminated pathogen in xylem vessels, which finally cause wilting of tree (Murata et al. 2005; Yamada and Sakaue 2007; Takahashi et al. 2010; Torii et al. 2010; Torii et al. 2011; Torii et al. 2014a, 2014b). Thus, disease management should be focused on the control of the pathogen (*Raffaelea quercus-mongolicae*) as well as insect vector (Jewell 1956). Many measures such as sticky roll trap, pheromone trap, above ground spray of insecticide, etc. were developed and applied. However, for the control of oak wilt in USA, a systemic fungicide, Alamo® (a.i., 14.5% propiconazole) was registered as an injectable fungicide and commercially available for the control of oak wilt caused by *Ceratocystis fagacearum* (Osterbauer and French 1992; Juzwik et al. 2011).

In this study endophytic and soil microorganisms were isolated from oak tree tissues and rhizosphere in Mongolian oak forest, and their antifungal activity was screened against oak wilt fungus. Selected isolates in the primary screening were compared in antifungal activity by culturing these isolates under various culture conditions. Mycelial growth, sporulation, and spore germination of oak wilt fungus were also compared on culture media supplemented with heated and unheated culture filtrates of antifungal microorganisms. For the development of convenient and efficient application type of antifungal microorganisms into the trees in forest, studies on formulation, preservation, and reviability of these microorganisms were conducted. The obtained results will provide fundamental information and contribute to the development of efficient biocontrol agent against oak wilt disease.

Materials and Methods

Isolation of endophytic and rhizospheric microorganisms from Mongolian oak forest

For isolating endophytic and rhizospheric microorganisms from Mongolian oak forest, plant samples from different parts of Mongolian oak (*Quercus mongolica* Fisch. ex Ledeb.) and rhizospheric soil samples were collected from Mongolian oak, in which insect vector (*Platypus koryoensis*) were entered into trunk but showing no wilt symptom, and from soils at 20-30 cm underground in forest located at Cheonggye-San, Sujeong-Ku, Seungnam-Si, Gyeonggi-Do, Korea.

For surface sterilization, all plant samples were washed in tap water, but root samples were washed again in distilled water with ultrasonicator (Branson Co), and then immersed into 70% ethanol (v/v) and 1% sodium hypochlorite (w/v) for 1 and 3 min, respectively. After blotting on paper towel, they were cut into small pieces in 3×3 mm size, placed on WA (Water Agar; agar 20 g, distilled water 1 L), and incubated at 25°C for 7 days. Growing hyphae from samples were sub-cultured on fresh PDA (Potato Dextrose Agar, Difco) at 25°C.

To isolate endophytic actinomycetes, the samples were prepared by the same method with endophytic fungi. After then, the segments were placed on WA including 10 ml of

Table 1. Chemical composition of HV agar for the isolation of soil actinomycetes

Chemical composition	Amount	Remarks
Humic acid	1.0 g	dissolve in 0.2N NaOH 10 ml
Sodium hydrogen phosphate dibasic	0.5 g	-
Potassium chloride	1.71 g	-
Magnesium sulfate	0.05 g	-
Ferrous sulfate heptahydrate	0.01 g	-
Calcium carbonate	0.01 g	-
B-vitamins	3.75 mg*	filter-sterilized
Cycloheximide	50 mg	filter-sterilized
Distilled water	1 L	

*0.5 mg each of thiamine-HCl, riboflavin, niacin, pyridoxin-HCl, inositol, Ca-pantothenate and p-aminobenzoic acid, and 0.25 mg of biotin.

**pH was adjusted 7.2 using NaOH.

Heritage (Syngenta; including azoxystrobin 50%), 25 mg of streptomycin sulfate (Sigma) and 10 ml of amphotericin B (Sigma; 50 mg dissolved in 10 ml of DMSO), and then incubated at 30°C for 7 days. The colonies were sub-cultured onto membrane filter (mixed cellulose ester, pore size 0.2 μm), which have pores only permeable to actinomycetes, placed on IMA-2 medium (glucose 5 g, soluble starch 5 g, beef extract 1 g, yeast extract 1 g, N-Z-case® 2 g, NaCl 2 g, $CaCO_3$ 1 g, agar powder 15 g and distilled water 1000 mL) and then incubated at 30°C. When actinomycetes grew out after 1 month or more of incubation, they were passed through membrane filter and sub-cultured on PDA.

The soil samples were air-dried for over 24 hrs until soil moisture evaporated. Ten gram of dried soil and 90 mL of sterilized water stir-mixed to make soil suspension and diluted serially from 10^{-3} to 10^{-7}. The diluted suspensions were spread on HV (Humic acid-Vitamin) agar (Table 1) and incubated at 30°C for 7 days. After 7 days, colonies of soil actinomycetes were observed with optical microscope and incubated at 30°C after transferring onto PDA with the sterilized toothpick. For making humic acid used in HV agar, the 500 g of soil samples were suspended in 1000 ml of 0.5% NaOH solution and maintained at room temperature for 24 hrs with occasional stirring. After centrifuge at 7,000 rpm for 20 min, precipitate of the suspension was removed and the supernatant was adjusted to pH 1.0 with HCl. The resulting precipitate was recovered by centrifugation at 3,000 rpm for 20 min, washed 3 times by centrifugation with approximately 150 mL of distilled water and then suspended again in 150 mL of distilled water. The suspension was frozen at -20°C overnight. After thawing it, the granulated humic acid was filtered off, washed and dried (Hayakawa and Nonomura 1987).

In vitro screening of the isolated microorganisms against oak wilt fungus

All isolated microorganisms were incubated on PDA for 7 days to screen their antifungal activity against oak wilt fungus, *R. quercus-mongolicae*. *R. quercus-mongolicae* isolate RQ 10.210 was supplied by the Division of Forest Insects and Diseases at the National Institute of Forest Science. The colony discs of all microorganisms were taken with sterile cork borer (∅ 8.5 mm), placed on the 4 edges of 90 mm PDA plate, and the agar discs containing mycelium of oak wilt fungus were placed on the center of same plate in the primary screening (Fig. 1). For the secondary screening, both isolated microorganism and oak wilt fungus were paired at opposite sides in the same plate. The plates were incubated at 25°C for 7 days, and inhibition zones were measured as an index of antifungal activity. In case of soil actinomycetes, MS (Mannitol Soya bean; 20 g of mannitol, 20 g of soya bean, 16 g of agar and 1,000 ml distilled water) and PDA were used for the primary and secondary screening, respectively.

Identification of the selected microorganisms

Antifungal isolates selected from primary and secondary screening were identified by molecular sequencing. To extract genomic DNA, all isolates were shake-cultured in PDB at 160 rpm and 30°C for 7 days. Spore and mycelium were harvested by using Miracloth, washed with distilled

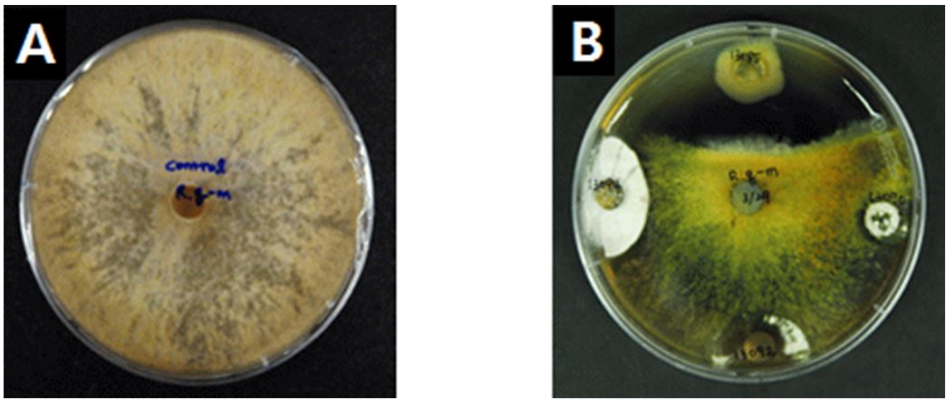


Fig. 1. (A) Colony morphology of oak wilt fungus, *Raffaelea quercus-mongolicae* on PDA, (B) Paired growth of oak wilt fungus, *R. quercus-mongolicae* with *Streptomyces* isolates. Mycelial growth of oak wilt fungus on the center was greatly inhibited by the one of *Streptomyces* isolates placed on the top of plate.

water and freeze-dried. After then, the colonies were disrupted and extracted with DNeasy Plant Mini Kit (QIAGEN Co.) according to the manufacturer's instructions and maintained at 4°C.

The 16S region of nuclear rDNA for actinomycetes was amplified by PCR using the primers 27F (5'-ATA GTT TGA TCM TGG CTC AG-3') and 1525R (5'-AAG GAG GTG WTC CAR CC-3'). The PCR condition were pre-denaturation of 5 min at 95°C, 30 cycles of 1 min at 95°C for denaturation, 1 min at 50°C for annealing and 1 min at 72°C for extension, and a final extension of 10 min at 72°C. Purification and sequencing of PCR products were performed by Macrogen® (Macrogen, Seoul, Korea). The primers used for sequencing were the same as used for the PCR reactions. The obtained sequences were analyzed with the SeqMan version 7.1.0 (DNASTAR, USA) and were performed to BLAST searches in NCBI/GenBank database (Ghobad-Nejhad et al. 2012).

Optimum culture condition for strong antifungal activity of antifungal isolates against oak wilt fungus

To find optimum culture condition of the selected isolates for strong antifungal activity, PDA and PDB were used as standard media.

For PDA culture, an agar disc containing mycelium was taken with cork borer (∅8.5 mm), inoculate on culture media by streaking with sterile spathula, and then cultured in the dark at 25°C and 30°C for 1 or 2 weeks. Agar discs (∅ 8.5 mm) of cultured mycelium of the selected isolates under various conditions and oak wilt fungus were dual-cultured at both sides on PDA supplemented with streptomycin sulfate at 100 ppm concentrations.

For PDB culture, an agar disc containing mycelium was taken with cork borer (∅8.5 mm), inoculate in PDB, and cultured in shaking incubator at 160 rpm under the same culture conditions. Culture suspension were centrifuged at 9,000 rpm for 20 min, the supernatants were decanted and the sterilized paper discs (∅8.5 mm) were dipped in the precipitate of mycelium and spores. Paper discs dipped in each precipitate of isolates cultured under various conditions and oak wilt fungus were paired at both sides on PDA supplemented with streptomycin sulfate at 100 ppm concentrations. All plates were kept in the dark at 25°C for 7 days, inhibition zone were measured by using caliper, and inhibition rates were calculated. Each treatment was carried out in 5 replications.

Preparation of culture media containing culture filtrate of antifungal microorganisms and antifungal activity against oak wilt fungus

After 5 *Streptomyces* isolates selected from the primary screening were cultured in the dark at 30°C for 7 days, mycelium harvested from the surface of culture media were inoculated in PDB and cultured in shaking incubator with 160 rpm speed at 25°C or 30°C for 1 or 2 weeks. Culture suspension were centrifuged at 9,000 rpm for 20 min, and culture filtrate were harvested by passing through membrane filter (pore size 0.45 μm).

To investigate heat sensitivity of antifungal metabolites in culture filtrate, 2 different culture media supplemented with autoclaved (heated) or unheated culture filtrate were prepared. For culture media with heated culture filtrate, the same volumes of PDA and culture filtrate were mixed with agar powder (7.5 g/L) before autoclaving at 121°C for 20 min. After cooling, streptomycin sulfate were added at 100 ppm concentrations before plating. Control culture media were prepared by mixing of PDA with distilled water instead of culture filtrate, and autoclaving. For culture media with unheated culture filtrate, PDA and agar powder (7.5 g/L) were autoclaved at 121°C for 20 min. After cooling, the media were mixed with the same volume of culture filtrate and streptomycin sulfate at 100 ppm concentrations before plating. Control culture media were prepared by mixing of PDA with sterilized water instead of culture filtrate.

To compare antifungal activity of heated or unheated culture filtrates against oak wilt fungus, agar disc (∅8.5 mm) of oak wilt fungus was placed on the center of culture media supplemented with heated or unheated culture filtrates, and then kept in the dark at 25°C for 5 days. Each treatment was carried out in 5 replications. Mycelial growths of oak wilt fungus were measured, and the inhibition rates of mycelial growth were calculated by the following formula. Inhibition rate (%)=[(mycelial growth of the control − mycelial growth of the treated)/mycelial growth of the control]×100

To compare the effects of heated or unheated culture filtrates on sporulation and spore germination of oak wilt fun-

gus, sporulation was stimulated by keeping culture plates in the dark and light alternation at room temperature 2 more days after 5 days' culture in the dark. Spores produced on the surface of culture media were harvested by adding sterile water, scrapping with cell scraper (SPL), and filtering through Miracloth (Merck Millipore Co.; Pore size: 22-25 μm). Number of spores was counted with Haemacytometer under compound microscope. Each experiment was carried out in 5 replications.

Spore suspensions of oak wilt fungus were spread on the surface of culture media supplemented with heated or unheated culture filtrates by using spreader (SPL). Germinated spores were counted under dissecting microscope at 18 hrs after treatment, and the inhibition rates of spore germination of the treated were calculated by comparing with control's. Each treatment was carried out in 3 replications.

Lyophilization of antifungal Streptomyces isolates and viability after storage

Five *Streptomyces* isolates showed strong antifungal activity against oak wilt fungus were cultured on PDA in the dark at 30°C for 5 days. Mycelium grown on the surface of culture media were harvested, inoculated in PDB, and shake-cultured in the dark with 160 rpm speed at 25°C for 10 days. Mycelium and spores were harvested by passing through Miracloth in clean bench, blended with sterilized water to make mycelial suspension. Twenty four % of skimmed milk sterilized at 110°C for 15 min. were mixed with the same volume of mycelial suspension to make mycelial suspension at the final concentration of 12% in test tube (Falcon), plugged with sterilized cotton, and subsequently stored at 4°C for more than 3 hrs, freezed at -20°C for more than 3 hrs and at -80°C for more than 6 hrs, lyophilized for 24 hrs, vacuum sealed and kept in the darkroom at 4°C.

For testing viability of the stored isolates, sterilized water were added, vortexed, 100 μL of suspension were plated and spread on PDA, and the growth of isolates were checked after incubating in the dark at 25°C for 7 days.

Results and Discussion

Isolation of endophytic and rhizospheric microorganisms from Mongolian oak forest

Three hundreds and thirty six of endophytic fungi were isolated from various parts of Mongolian oak tree. Isolation frequencies of endophytic fungi from heartwood, sapwood, root, branch, twig, and leaf were 39.7, 34.2, 14.9, 5.7, 3.9, 1.5%, respectively. In contrast, 10 endophytic actinomycetes were isolated from sapwood and root samples, while 255 actinomycetes were isolated from rhizospheric soil samples (Table 2).

In vitro screening of the isolated microorganisms against oak wilt fungus

All endophytic actinomycetes did not show the antifungal activity against oak wilt fungus. Among 255 actinomycetes from rhizospheric soil samples, 9 isolates showed relatively strong antifungal activity with 7.5-13.5 mm of inhibition zone (Table 3). Two isolates, TPML13085 and 13086 showed the strongest antifungal activity. Of 9 isolates, 5 isolates (13085, 13086, 13089, 13092, 13093) were selected and used for the additional studies.

Table 2. Isolation frequency of endophytic and rhizospheric microorganisms from different parts of Mongolian oak tree and rhizospheric soil samples in oak forest

Parts of Mongolian oak tree		Endophytic fungi	Endophytic actinomycetes	Soil actinomycetes
Trunk				
Heartwood	50*	64 (19.0%)		-
	100	17 (5.0%)	-	-
	150	25 (7.4%)	-	-
	200	28 (8.3%)	-	-
Sapwood	50	50 (14.9%)	2 (20%)	-
	100	12 (3.6%)	1 (10%)	-
	150	28 (8.3%)	-	-
	200	25 (7.4%)	2 (20%)	-
Root				
Taproot		39 (11.6%)	4 (40%)	-
Rootlet		11 (3.3%)	1 (10%)	-
Branch		19 (5.7%)	-	-
Twig		13 (3.9%)	-	-
Leaf		5 (1.5%)	-	-
Soil		-	-	255 (100%)
Total		336 (100%)	10 (100%)	255 (100%)

*The height of trunk from the soil line, at which the samples were collected.

Identification of the selected microorganisms

All selected actinomycetous isolates with strong antifungal activity were identified as *Streptomyces* species by 16S region of rDNA sequencing analyses. TPML13085, 13086, 13087, and 13089 were identified as the same species *S. blastmyceticus*, TPML13090 as *S. sanglieri*, TPML13091 as *S. sannanensis*, TPML13092 as *Streptomyces* sp., TPML13093 as *S. xanthochromogenes*, and TPML13094 as *S. tsukiyonensis*, respectively (Table 3).

Optimum culture condition for strong antifungal activity of antifungal isolates against oak wilt fungus

In all culture conditions, 3 isolates of *S. blastmyceticus* (TPML13085, 13086, 13089) showed stronger antifungal activity against oak wilt fungus than *Streptomyces* sp. (TPML13092) and *S. xanthochromogenes* (TPML13093). Cultures of them in PDB at 30°C for 1 week showed slightly stronger antifungal activity than cultures on PDA at 25°C for 2 weeks. Among 3 isolates of *S. blastmyceticus*, TPML13085 showed the highest mycelial growth inhibition rate (46.1%) of oak wilt fungus when it was cultured in PDB at 25°C for 1 week. Culture conditions did not show any differences in mycelial growth inhibition rate for *Streptomyces* sp. (TPML13092) and *S. xanthochromogenes* (TPML13093) (Table 4).

Table 3. *Streptomyces* isolates tested in the secondary screening, which showed strong antifungal activity against oak wilt fungus in the primary screening

Strain No.	Scientific name	Antifungal activity
TPML13085	*Streptomyces blastmyceticus*	13.5
TPML13086	*Streptomyces blastmyceticus*	13.5
TPML13087	*Streptomyces blastmyceticus*	10.4
TPML13089	*Streptomyces blastmyceticus*	12.6
TPML13090	*Streptomyces sanglieri*	7.5
TPML13091	*Streptomyces sannanensis*	11.7
TPML13092	*Streptomyces* sp.	8.1
TPML13093	*Streptomyces xanthochromogenes*	12.3
TPML13094	*Streptomyces tsukiyonensis*	12.3

Antifungal activity against oak wilt fungus on culture media containing culture filtrate of antifungal isolates

Mycelial growth inhibition rates (MGIRs) of 3 *S. blastmyceticus* isolates cultured on media supplemented with heated culture filtrate at 25°C and 30°C for 2 weeks were 80.7%, 72.4% for TPML13085, 82.2%, 75.0% for TPML13086, and 15.5%, 59.7% for TPML13089, respectively. Generally, inhibition rates of isolates cultured for 2 weeks were much higher than those cultured for 1 week. It means that antifungal metabolites may be produced more after 1 weeks from the beginning of culture.

Average MGIR of 3 *S. blastmyceticus* isolates cultured on media supplemented with unheated culture filtrates were very high as 91.3% in all culture conditions. When these 3 isolates were cultured at 30°C for 2 weeks, all isolates completely inhibited mycelial growth of oak wilt fungus, while MGIRs were 97.6% for TPML13085, 90.6% for TPML13086, and 100% for TPML13089 when cultured at 25°C for 2 weeks. Mycelial growth inhibition rates (MGIR) of 3 *S. blastmyceticus* isolates cultured on media supplemented with unheated culture filtrate at 25°C and

Table 4. Inhibition rate (%) of *Streptomyces* isolates cultured in different conditions against oak wilt fungus by *in vitro* pairing test

Culture conditions	*Streptomyces* isolates	13085		13086		13089		13092		13093	
Temperature (°C)	Period (weeks)	PDA	PDB	PDA	PDB	PDA	PDB	PDA	PDB	PDA	PDB
25	1	40.6	46.1	40.8	40.9	39.1	42.0	28.0	28.9	32.7	31.7
	2	36.6	37.7	33.8	35.8	35.0	38.9	27.6	27.0	24.4	27.6
30	1	41.6	41.5	42.9	41.3	43.0	42.5	29.1	27.2	34.8	33.0
	2	38.4	40.6	34.0	36.5	41.9	40.5	26.4	23.8	31.9	28.9
Mean		39.3	41.5	37.9	38.6	39.8	41.0	27.8	26.7	30.9	30.3

PDA, Potato Dextrose Agar; PDB, Potato Dextrose Broth.

30°C for 1 weeks were 81.1%, 88.4% for TPML13085, 83.4%, 84.9% for TPML13086, and 79.3%, 90.7% for TPML13089, respectively. In all culture conditions, MGIRs of antifungal isolates cultured on media with unheated culture filtrate were higher than those cultured on media with heated culture filtrate. These results means that antifungal metabolites of isolates might be greatly inactivated by heating. *Streptomyces* sp. (TPML 13092) and *S. xanthochromogenes* (TPML 13093) showed relatively very low or almost zero MGIR in all culture conditions (Table 5).

Sporulation inhibition rates (SIRs) of *S. blastmyceticus* isolate (TPML13085) were relatively higher when the isolate was cultured on media with heated culture filtrate for 1 week than for 2 weeks. SIRs on media with unheated culture filtrate was relatively higher on media with unheated culture filtrate at 30°C than 25°C (Table 6).

Spore germination of oak wilt fungus on culture media supplemented with heated culture filtrate of TPML13085 cultured at 25°C or 30°C for 2 weeks was completely inhibited, while inhibition rate was 82% on the media with the culture filtrate cultured at 25°C for 1 week. Unexpectedly,

Table 5. Mycelial growth inhibition rate (%) of oak wilt fungus (RQ10.210) on culture media supplemented with heated and unheated culture filtrates of antifungal *Streptomyces isolates*

Culture condition	*Streptomyces* isolates	13085		13086		13089		13092		13093	
Temperature (°C)	Period (weeks)	H	U	H	U	H	U	H	U	H	U
25	1	0.0	81.1	0.0	83.4	0.0	79.3	0.0	0.0	0.0	0.0
	2	72.4	97.6	82.2	90.6	15.5	100.0	0.0	0.0	0.0	0.0
30	1	49.2	88.4	0.0	84.9	0.0	90.7	0.0	0.0	0.0	0.0
	2	80.7	100.0	75.0	100.0	59.7	100.0	0.0	0.0	19.0	45.9
Mean		50.6	91.8	39.3	89.7	18.8	92.5	0.0	0.0	4.8	11.5

Culture filtrates were obtained by collecting supernatants after centrifugation of culture suspension, and then filtered through membrane filter (0.45 μm). Test media were made of mixtures of the equal volumes of fresh culture filtrate and culture media. Each data represent the mean of 5 replicates. H, heated; U, unheated culture filtrate.

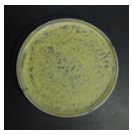
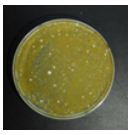
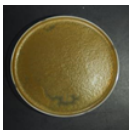
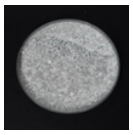
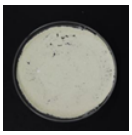
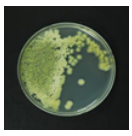
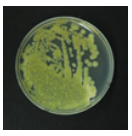
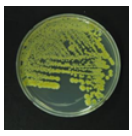
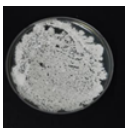
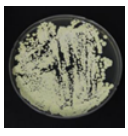
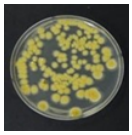
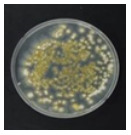
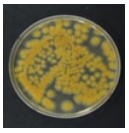
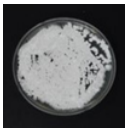
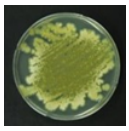
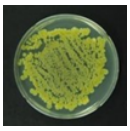
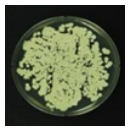
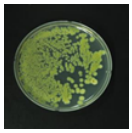
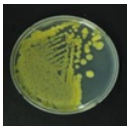
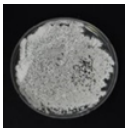
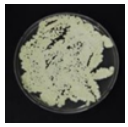
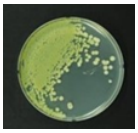
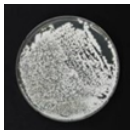
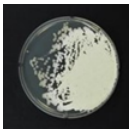
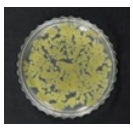
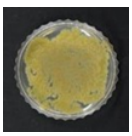
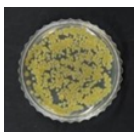
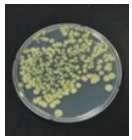
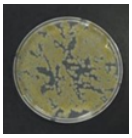
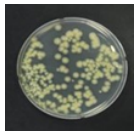
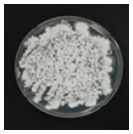
Table 6. Inhibitory effects of heated and unheated culture filtrates of antifungal *Streptomyces blastmyceticus* (TPML13085) on sporulation and spore germination of oak wilt fungus

Culture filtrate	Culture temperature (°C)	Culture period (weeks)	Inhibition rate (%)*	
			Sporulation[1)]	Spore germination[2)]
Heated	25	1	58.0	82.1
		2	0	100.0
	30	1	62.9	0
		2	10.5	100.0
Unheated	25	1	0	100.0
		2	0	100.0
	30	1	11.7	100.0
		2	100.0	100.0

*Inhibition rate (%) of sporulation = [(No. of spores for the control − No. of spores for the treated)/No. of spores for the control] × 100.
*Inhibition rate (%) of spore germination = [(No. of germinated spores for the control − No. of germinated spores for the treated)/ No. of germinated spores for the control] × 100.
[1)]Sporulation of the control on culture media with heated and unheated culture filtrates were 1.43×10^6/mL, and 0.6×10^6/mL, respectively.
[2)]Spore germination of the control on culture media with heated and unheated culture filtrates were 28 and 31, respectively.

the inhibition of spore germination was not observed on the media with the culture filtrate cultured at 30°C for 1 week. This result may be a kind of error resulting from culture contamination, and need to be checked again. In case of the test on culture media supplemented with unheated culture filtrate, spore germination was completely inhibited in all culture conditions. These results indicate that antifungal metabolites filtered from TPML13085 culture have a strong inhibition activity against mycelial growth, sporulation and spore germination of oak wilt fungus, *R. quercus-mongolicae*, but some of them were heat-sensitive and generally lost antifungal activity partially when the culture

Table 7. Re-viability of lyophilized mycelium of *Streptomyces* isolates on PDA plate after storage at 4°C

Storage Period	Streptomyces isolates				
	13085	13086	13089	13092	13093
0 month					
1 month					
2 months					
3 months					
4 months					
5 months					
6 months					
7 months					
8 months					

filtrate was autoclaved. From the above results, for the development of biocontrol agent against oak wilt fungus, further researches based on the secondary metabolites and genetic analyses of antifungal microorganisms against fungal pathogens are strongly required to be carried out (Hopewood 1967; Hopwood et al. 1973; Chater et al. 1982; Hopewood 1988; Chater 1989).

Viablity of antifungal Streptomyces isolates after lyophilization and storage

S. blastmyceticus (TPML13085, 13086, 13089), *Streptomyces* sp. (TPML13092), *S. xanthochromogenes* (TPML13093) were viable until 240 days' storage after lyophilization of spores and mycelium (Table 7). This result could be applied for developing long term storage of the isolates and for improving formulation technology, which is related to the ease-of-use in field application.

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