Journal of Plant Protection Research ISSN 1427-4345

ORIGINAL ARTICLE

Damping-off disease reduction using actinomycetes that produce antifungal compounds with beneficial traits

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Vol. 60, No. 3: 233–243, 2020

DOI: 10.24425/jppr.2020.133318

Received: September 4, 2019
Accepted: February 4, 2020

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Abstract

Actinomycetes are considered to be the biggest producer of bioactive compounds which are expected to have antifungal activity for controlling many fungi such as *Rhizoctonia solani*. The objective of this study was to obtain potential soybean rhizosphere actinomycetes as a biocontrol agent for *R. solani* which cause damping-off disease both *in vitro* and *in vivo*, including their ability to produce siderophore, chitinase, and HCN. Out of 26 isolates, 18 (56%) showed diverse antifungal activities against *R. solani* with percentages of inhibition radial growth (PIRG) from 18.9 to 64.8%, as evaluated by a dual culture method. Ten isolates with the strongest antifungal activity were numbered for further characterization. All the tested isolates were not antagonistic towards *Bradyrhizobium japonicum*. These isolates were able to suppress damping-off disease caused by *R. solani* in the greenhouse experiment. Isolate ASR53 showed the highest disease suppression, 68% and 91% in sterile and non-sterile soil, respectively. Based on 16S rRNA sequence analysis this isolate belonged to *Streptomyces violaceorubidus* LMG 20319 (similarity 98.8%) according to GenBank data base available at www.ncbi.nlm.gov.nih. Furthermore, isolate ASR53 had significantly longer roots and shoots, as well as greater fresh and dry weights of seedlings than the control. Crude extract derived from ASR53 isolates contained 10 dominant compounds that were biologically active against fungal pathogens. Thus, this study suggests that the application of potential actinomycetes of the soybean rhizosphere can act as a promising biocontrol agent against damping-off disease caused by *R. solani.*

Keywords: antifungal activity, disease suppression, *in planta*, *Streptomyces*, root rot

Introduction

Rhizoctonia solani Kuhn [teleomorph, *Thanatephorus cucumeris* (Frank) Donk] are phytopathogenic fungi which have destructive effects and cause global damage of more than 142 plant species, including agricultural and horticultural plants (Mishra *et al.* 2014). Soybean is one of many plants vulnerable to infection with *R. solani*. The damping-off disease has certain symptoms, such as root rot, brown stem lesions, shrunken stems, withered and dead plants which drop soybean productivity (Gonzales *et al.* 2011). In Indonesia, damping-off disease invasion can cause yield losses of up to 100% if the pathogen attack is not controlled during the early phases of soybean growth (Khaeruni and Rahman 2012). The disease can spread easily during the rainy season and with high humidity. The pathogen can survive in the soil by forming sclerotia and dormant mycelium which can survive in plant debris (Feng *et al.* 2017).

Damping-off disease is managed by cultural practices, such as crop rotation, the use of chemical fungicides, soil solarization and minimizing prolonged contact of the plant with pathogens by planting in the

dry season and removing the remaining tubers from the fields (Helal 2017). Current cultural methods and chemical controls are not entirely effective, and damping-off disease remains a persistent problem. Therefore, environmentally safe biocontrol agents need to be explored.

Actinomycetes are Gram-positive bacteria widely distributed in many habitats. The most dominant genus found in soil is *Streptomyces*. Other genera such as *Nocardiopsis, Saccaromonospora, Amycolaptosis, Actinoplanes*, and *Catenuloplanes* are frequently found in soil (Ratnakomala *et al.* 2016; Retnowati *et al.* 2017). The filamentous morphology of actinomycetes makes them effective in colonizing the plant rhizosphere. As a rhizobacterium, actinomycetes influence plant growth by both direct and indirect mechanisms. In the direct mechanism, they can produce phytohormones (Khamna *et al.* 2010) and solubilize phosphate (Hamdali *et al.* 2012). Whereas in the indirect mechanism, they have been reported to have the ability to produce siderophore, hydrogen cyanide acids (HCN), cellulase, and protease (Gopalakrishnan *et al.* 2011). For plant defense against the pathogen, actinomycetes also produce antibiotic and antifungal compounds (Harsonowati *et al.* 2017; Lyu *et al.* 2017), as well as hydrolytic enzymes such as chitinase (Shivalee *et al.* 2018), and β-1,3-glucanase (Shreevidya *et al.* 2016).

The role of actinomycetes as biocontrol agents against fungi should be explored for reducing disease incidence caused by *Fusarium* spp. (Gopalakrishnan *et al.* 2011), *Pythium* sp. (Costa *et al.* 2013), *Rhizoctonia* spp. (Sadeghi *et al.* 2009). The destructive effects of metabolites and lytic enzymes produced by actinomycetes are possibly involved in cell wall lysis, thinning of hyphae, discoloration of hyphae, and inhibition of germ tube elongation (Prapagdee *et al.* 2008; Kaur *et al.* 2016).

In this study, 26 isolates of actinomycetes were isolated from soybean rhizosphere soil from Sukabumi, West-Java, Indonesia. Some of them were shown to be capable of promoting plant growth through several mechanisms such as producing indole-3-acetic acid (IAA), solubilizing phosphate, fixing nitrogen, and enhancing soybean germination via *in vitro* assay. The aim of this study was to recover potential soybean rhizosphere actinomycetes which produce antifungal compounds that could be used as biocontrol agents for *R. solani* damping-off disease both *in vitro* and *in planta*. The application of actinomycetes isolated from the soybean rhizosphere is expected to protect soybean from damping-off disease and to promote soybean growth. To raise soybean yield the findings of this research can be utilized to provide an ecologically friendly fungal biocontrol agent, especially for damping-off disease.

Materials and Methods

Actinomycete strains and *Rhizoctonia solani* culture

A total of 26 isolates of actinomycetes isolated from the soybean rhizosphere in a previous study (collection of the Microbiology Laboratory, at the Department of Biology, Mathematics and Natural Science Faculty, Bogor Agricultural University, Indonesia) were used. Strains were cultured on International Streptomyces Project (ISP) 2 medium [4 g · l^{-1} yeast extract, 10 g · l^{-1} malt extract, 4 g · l^{-1} dextrose, and 20 g · l^{-1} agar (Himedia, Mumbai, India)] and incubated at 28°C for 4 days. *Rhizoctonia solani* was obtained from the Laboratory of Clinical Plants at the Department of Plant Protection, Bogor Agricultural University, Indonesia and cultured on potato dextrose agar (PDA) (Himedia, Mumbai, India).

Screening for antifungal activity of actinomycetes *in vitro*

All 26 isolates were used for screening of their antifungal activity against *R. solani*. This experiment was conducted with a dual culture assay using PDA medium. Streptomyce isolates were streaked on the edge of the PDA plate (25 mm from the edge of the plate). After 2 days of incubation, an agar-mycelium disc (8 mm in diameter) of *R. solani* was inoculated in the center of the plate. The plates were then incubated for 3 days at 24°C (Bonaldi *et al.* 2014). Three replicates were prepared for each treatment. The percentage of inhibition radial growth (PIRG) of *R. solani* was calculated using the following formula by Wiraswati *et al.* (2019):

$$\text{The Percentage of Inhibition Radial Growth} = \frac{B-A}{B} \times 100\ [\%],$$

where: *A* – shows the distance between a fungus' center point toward the actinomycete isolates, *B* – shows the distance between a fungus' center point towards the opposite side without bacteria.

Rhizobial compatibility test

Ten isolates with antifungal activity were tested for rhizobial compatibility. This assay was conducted to assess the interaction between actinomycetes and *Bradyrhizobium japonicum* strain BJ 11 (collection of the Microbiology Laboratory of IPB), based on methods described by Zanatta *et al.* (2007) with a slight modification of the bacterial medium culture. Modified Luria Agar (LA modified) medium

(5 g · l^{-1} yeast extract, 10 g · l^{-1} casein, 0.5 g · l^{-1} NaCl, and 20 g · l^{-1} agar) was inoculated with 8% of 4-day-old *B. japonicum,* then poured into a Petri dish and used as a basal layer. Twenty microliters of actinomycete culture (7-days old) was then inoculated onto the paper disc in that inoculated medium. The plates were then incubated at 25°C for 7 days, and antagonism was detected from the clear zone formation. This assay was done in triplicate.

Evaluation of disease suppression of actinomycetes under greenhouse conditions

The evaluation of disease suppression was prepared in three main steps: the preparation of the fungal pathogen, preparation of the actinomycete inoculum, and a greenhouse experiment, as described below.

Inoculum preparation for pathogenic fungi

Fungal propagation was done according to Susilowati *et al.* (2011). The *R. solani* inoculum was prepared by inoculating one plug of fungal culture in 100 ml of potato dextrose broth (PDB) supplemented with 50 µg · ml^{-1} rifampicin. The culture was agitated at 110 rpm for 7 days. Fungal mycelium was collected by filtering and washed twice using sterile distilled water. The filtrate was weighed and homogenized using a blender. The number of inoculum (CFU · ml^{-1}) was determined by the total plate count method. The fungal pathogen population of about 10^3 CFU · g^{-1} was then inoculated into the soil.

Seed coating preparation

Seed coating was done according to Susilowati *et al.* (2011) and Amini *et al.* (2016). Soybean seeds (cv. Wilis) were surface-sterilized in 2% NaClO for 3 min and further washed five times with sterile distilled water $2H_2O$. Actinomycetes with antagonist properties were cultured on ISP2 medium for 10 days at 28°C. Aerial mycelium was scraped and collected into a test tube and soaked in 0.85% NaCl. The homogenized spore suspension (10^8 CFU · ml^{-1}) was centrifuged and washed twice with sterile distilled water. A spore pellet was collected and suspended with 0.5% carboxymethyl cellulose (CMC). The sterilized seeds were soaked in the spore suspension for 30 min and shaken vigorously. Control seeds were treated only with 0.5% of CMC. Treated seeds were planted in sterile and non-sterile soil in pots infested with 30 ml of *R. solani* culture (10^3 CFU · g^{-1}).

Soil preparation and greenhouse experiment

Soil (ultisol soil, sand, and organic compost at a ratio of 2 : 1 : 1) was sterilized for 2 consecutive days at 121°C for 1 h. Soybean seeds were soaked in actinomycete spore suspension, then sown in a pot at a depth of 1 cm and covered with the infected fungal soil. The three experimental groups consisted of: (1) one non-infested soil with the non-inoculated seed (negative control), (2) *Rhizoctonia solani* infested soil with non-inoculated seed (positive control), and (3) *Rhizoctonia solani* infested soil with inoculated seed. Each treatment was tested both in sterile and non-sterile soil in triplicate. Each replication consisted of 10 soybean seeds which had been soaked in actinomycete spore suspension. Seven days after sowing, the incidence of damping-off disease and disease suppression of antagonist actinomycetes was evaluated. The percentages of disease incidence were calculated by counting the number of infected plants compared to all plants in each treatment. The disease suppression was evaluated by using the following formula as described by Susilowati *et al.* (2011):

$$\text{Disease Suppression} = \frac{C^x - C^+}{C^- - C^+} \times 100\ [\%],$$

where:

C^x – the number of healthy plants in the treatment;
C^+ – the number of healthy plants in infected control;
C^- – the number of healthy plants in non-infected control.

The lengths of the shoots and roots from healthy plants were measured and weighed to determine fresh weight. The plants were dried overnight at 60°C to obtain the dry weight. The data were analyzed by one-way analysis of variance (ANOVA) and significant differences between means were compared using the Tukey test at p-value = 0.05.

Molecular identification based on 16S rRNA gene analysis

The highest disease suppression isolate was identified based on the 16S rRNA gene. The actinomycete genome was extracted by a bacterial isolation kit (Presto™ Mini gDNA Bacteria Kit from Geneaid, Taiwan), following the manufacturer's protocol. The 16S rRNA gene was amplified using specific primers for actinomycetes 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 16Sact1114R (5'-GAG TTG ACC CCG GCR GT-3') (Martina *et al.* 2008). The PCR reaction was performed in a total volume of 50 µl, containing 25 µl of DNA polymerase enzyme GoTaq Green Master Mix 2X, 2.5 µl lof each reverse and forward primer (10 pmol · $µl^{-1}$), 15 µl of nuclease-free water, and 5 µl of DNA template (100 ng · $µl^{-1}$). The PCR was done in 30 cycles, and this condition served for pre-denatu-

ration (95°C, 4 min), denaturation (95°C, 30 s), annealing (55°C, 30 min), elongation (72°C, 1 min), and post-elongation (72°C, 7 min). The PCR products were then visualized by electrophoresis using 1% agarose gel and migration at 80 V for 50 min. The gel was then stained with 1% of ethidium bromide (EtBr) for 15 min and observed under a UV transilluminator. The sequences were aligned by using Basic Local Alignment Search Tool-program for Nucleotide (BlastN) from the website of the National Center for Biotechnology Information (NCBI). A phylogenetic tree was then constructed using the neighbor-joining (NJ) method with 1000X bootstrap in MEGA 8.0 software.

An antifungal compound of crude extract

The actinomycete isolate with the highest antifungal activity pathogen in the greenhouse experiment, *Streptomyces* sp. ASR53, was inoculated in 1,000 ml of ISP2 broth and incubated in a rotary shaker for 10 days at 28 ± 2°C. The actinomycete culture was extracted with ethyl acetate (EtOAc) at a ratio of 1 : 1 (v/v). The organic phase of EtOAc was separated from the liquid medium using a decantation funnel, and evaporated in a rotary vacuum evaporator. To identify the chemical compound in the crude extract from *Streptomyces* sp. ASR53, GC-MS analysis was conducted. A HP-5MS (5% phenyl methyl siloxane) capillary column (250 µm × 30 µm × 0.25 µm) and 1 ml · min^{-1} helium was used as a carrier gas. The column temperature was initially set at 40°C for 10 min, followed by an increase of 3°C · min^{-1} to 250°C. The temperature then was set isothermally for 5 min. The MS was operated at 70 eV. The compounds were identified by comparison of their mass spectral data with those from WILEY09TH.L Spectral Library.

Results

In vitro antifungal activity

Eighteen isolates out of 26 showed antifungal activity against *R. solani* (Table 1) after 5 days of incubation. Among them, 10 isolates with PIRG values of more than 40% were used for the greenhouse experiment. The potential isolates were coded as isolates: ASR46, ASR47, ASR53, ASR54, ASR56, ASR58, ASR65, ASR67, ASR75 and ASR76. The PIRG values of the 10 isolates ranged from 40.2 to 64.8%. Isolate ASR53 exhibited the highest antifungal activity (64.8%), while isolate ASR65 showed the lowest antifungal activity in reducing the radial growth of *R. solani*. Hyphal inhibition of *R. solani* by *Streptomyces* sp. ASR53 *in vitro* on potato dextrose agar is shown in Figure 1.

Table 1. Inhibition activity by actinomycetes from the soybean rhizosphere towards *Rhizoctonia solani* fungal growth on PDA medium agar using dual culture assay

No.	Isolate	PIRG* ± SD
1	ASR53	64.8 ± 4.2
2	ASR46	53.0 ± 11.0
3	ASR54	52.8 ± 4.1
4	ASR67	46.7 ± 5.8
5	ASR56	46.5 ± 2.8
6	ASR47	46.2 ± 2.4
7	ASR58	43.0 ± 9.9
8	ASR76	43.4 ± 4.8
9	ASR75	40.1 ± 7.8
10	ASR65	40.2 ± 4.8
11	ASR44	38.7 ± 10.1
12	ASR78	37.4 ± 0.9
13	ASR72	36.4 ± 12.3
14	ASR45	33.3 ± 6.7
15	ASR55	31.3 ± 4.6
16	ASR52	24.4 ± 3.8
17	ASR64	20.0 ± 6.4
18	ASR41	18.9 ± 3.8

*PIRG – percentage of inhibition radial growth of the fungi by the isolate. Data in the table are means of three replicates ± standard deviation of the means

Compatibility isolates towards rhizobial bacteria

The objective of the compatibility assay was to investigate the interaction between actinomycetes and the *B. japonicum* strain BJ11. Based on this assay, all the tested isolates were not antagonist towards rhizobium since there was no clear zone formed around the actinomycete disc.

Greenhouse experiment

Ten potential rhizosphere actinomycete isolates were examined for their disease reduction activity in sterilized and non-sterilized soil in the greenhouse. Positive control treatment (pathogenic soil without antagonistic isolates) indicated the highest disease incidence in sterilized and non-sterilized soil with percentages of 76.7 and 63.3%, respectively. Seed coating treatment with antagonistic isolates and synthetic fungicide significantly decreased disease incidence ($p < 0.05$). The lowest disease incident level was detected from isolate ASR53 treatment with 20% in sterilized soil and 6.7% in non-sterilized soil (Fig. 2). The lower disease incidence from each treatment meant that actinomycetes

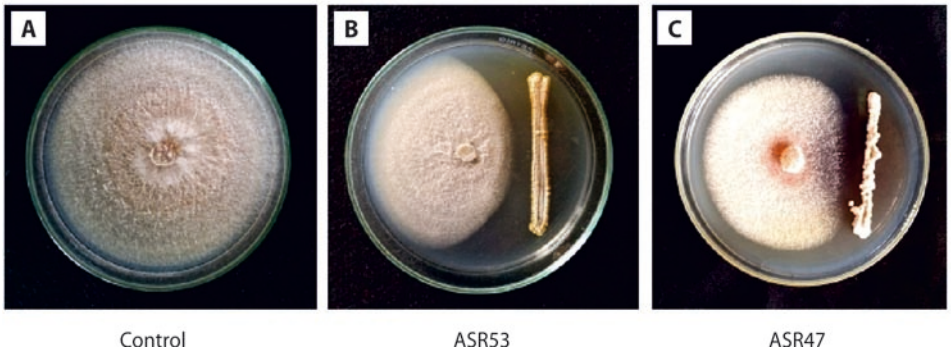

Fig. 1. Inhibition of *Rhizoctonia solani* by *Streptomyces* sp. ASR53 *in vitro* on potato dextrose agar. A – *R. solani* without actinomycetes as a control, B – antifungal activity of isolate ASR53, C – antifungal activity of isolate ASR47

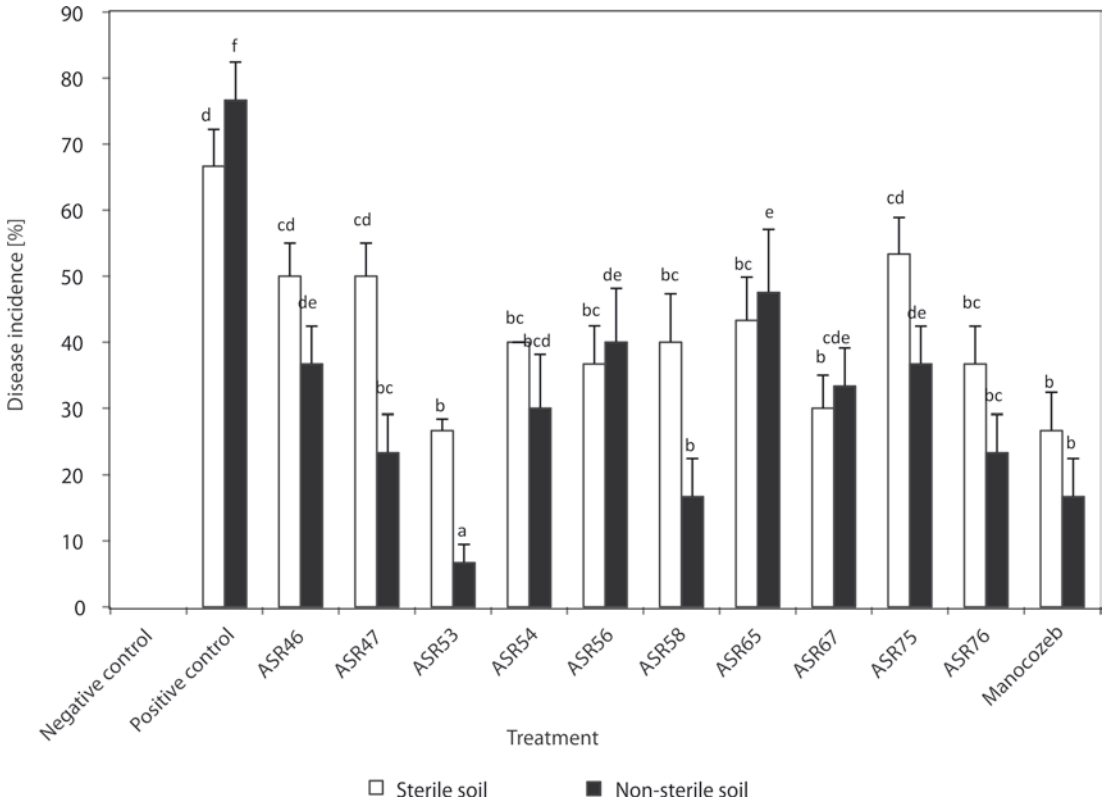

Fig. 2. Disease incidence symptoms of soybean damping-off after treated with seed coating actinomycetes spores on sterile soil and non-sterile soil. Bars sharing the same letter are not significantly different according to Tukey's honestly significant difference test at $p \leq 0.05$

were better able to suppress the disease. The two highest disease suppressions in sterilized soil were shown by isolates ASR53 and ASR58 with percentages of 91.1 and 77.9%, respectively. Furthermore, in non-sterilized soil, ASR53 and ASR67 revealed the highest inhibition activity of damping-off disease with percentages of 68.3 and 65.5%, respectively. Generally, the capacity of the actinomycetes to suppress *R. solani* damping-off disease in non-sterilized soil was relatively higher than in sterilized soil. Isolate ASR53 showed the highest efficacy in reducing the damping-off disease in sterilized and non-sterilized soil. The suppression by ASR53 was also higher than synthetic fungicide treatment, but it was not statistically significant (Fig. 3).

Plant growth parameters of soybean plants treated with antagonist actinomycetes are presented in Tables 2 and 3. The results indicated that soybean coated with actinomycete spores tend to show a better growth response than the pathogenic control. The results showed that between treatments there were significant differences in the lengths of shoots, and roots, and dry and fresh weights of plants ($p < 0.05$). Actinomycete treatment

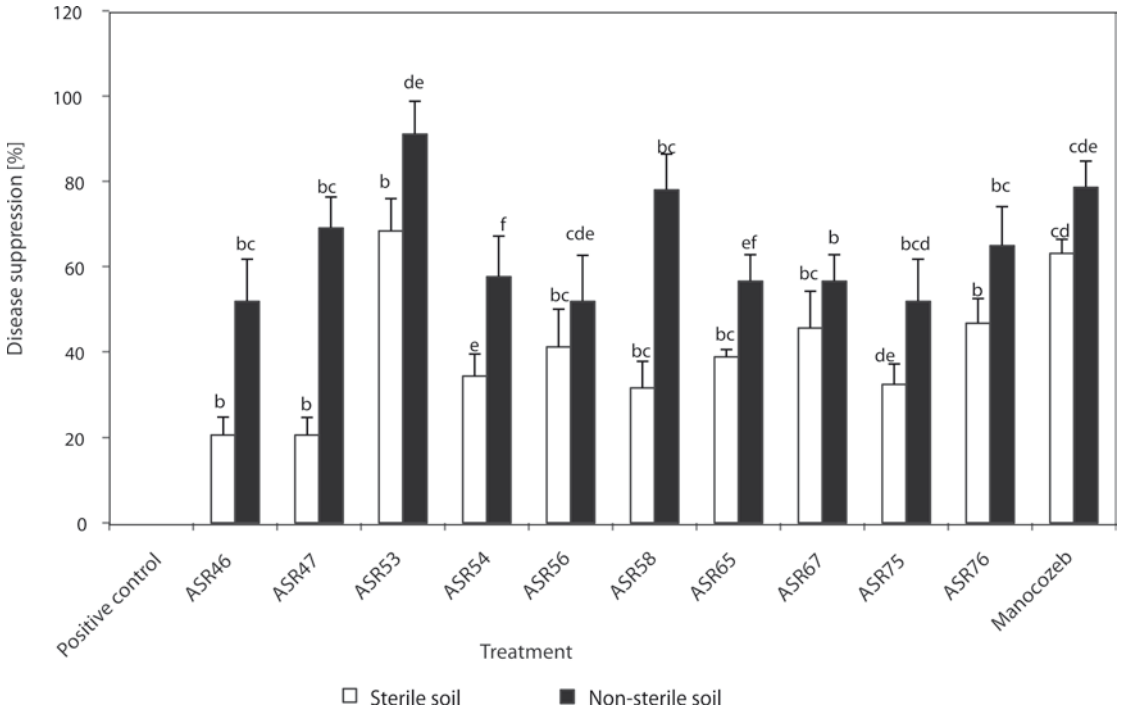

Fig. 3. Soybean damping-off disease suppression with seed coating actinomycetes spores and synthetic fungicides. Bars sharing the same letter are not significantly different according to Tukey's honestly significant difference test at $p \leq 0.05$

Table 2. Effect of selected actinomycetes on the growth of soybean in non-sterile soil amended with *Rhizoctonia solani*

Isolates	Growth parameters of actinomycete treated soybean			
	shoot length [cm]	root length [cm]	seedling fresh weight [mg]	seedling dry weight [mg]
Positive control	8.0 ± 0.7 a	5.2 ± 0.8a	548.0 ± 50.9 a	90.8 ± 3.1 a
ASR46	12.1 ± 0.4 b	8.8 ±1.0 cd	793.2 ± 98.7 cde	106.5 ± 7.6 ab
ASR47	13.8 ± 0.5 b	8.1 ± 0.4 bcd	739.4 ± 34.1 bcd	101.9 ± 7.9 ab
ASR53	12.1 ± 1.0 b	8.2 ± 0.2 bcd	893.5 ± 58.5 e	110.7 ± 4.0 bc
ASR54	12.4 ± 0.3 b	9.6 ± 0.5 d	797.1 ± 70.1 cde	102.8 ± 6.9 ab
ASR56	11.0 ± 0.2 bc	8.1 ± 0.3 bcd	651.7 ± 38.4 abc	104.0 ± 5.9 ab
ASR58	12.1 ± 0.5 b	9.3 ± 0.6 d	648.5 ± 13.5 abc	100.0 ± 5.8 ab
ASR65	10.8 ± 1.4 bc	8.0 ± 0.5 bcd	694.3 ± 39.4 abc	94.2 ± 8.9 ab
ASR67	12.2 ± 2.6 b	6.5 ± 0.6 ab	809.6 ± 22.0 de	93.8 ± 4.9 ab
ASR75	11.1 ± 1.7 bc	8.2 ± 0.3 bcd	754.0 ± 26.3 bcde	104.0 ± 4.5 ab
ASR76	11.6 ± 0.8 bc	8.6 ± 1.2 cd	620.2 ± 31.2 ab	94.5 ± 1.5 ab
Maconzeb	11.4 ± 0.7 bc	7.2 ± 1.0 bc	775.8 ± 14.7 cde	97.0 ± 1.0 ab

The different letters indicate the data were significantly different as analyzed by Tukey test ($p \leq 0.05$)

in sterilized soil had a significant effect on the lengths of shoots and roots and the dry weights of plants. Seed coating with ASR53 in sterilized soil and non-sterilized soil showed better growth than the pathogenic control and synthetic fungicide treatment. In non-sterilized soil, ASR53 significantly increased ($p < 0.05$) the length of plant shoots from 8.0 to 12.1 cm, the length of roots from 5.2 to 8.2 cm, fresh weight from 548 to 893 mg · plant^{-1}, and dry weight from 90.8 to 110.7 mg · plant^{-1}. A significant increase in soybean growth ($p < 0.05$) with isolate ASR53 treatment was also seen in sterilized soil plants: the shoot length from 9.9 to 11.5 cm, the root length from

Table 3. Effect of selected actinomycetes on the growth of soybean in sterile soil amended with *Rhizoctonia solani*

Isolates	Growth parameters of actinomycete treated soybean			
	shoot length [cm]	root length [cm]	seedling fresh weight [mg]	seedling dry weight [mg]
Positive control	9.9 ± 0.8 ab	2.7 ± 0.3 a	522 ± 20.6 a	83.2 ± 3.4 ab
ASR46	10.7 ± 0.1 b	4.4 ± 0.2 c	705 ± 55.2 def	112.8 ± 8.4 cd
ASR47	10.3 ± 0.5 ab	6.1 ± 0.3 fg	685 ± 41.0 cdef	90.2 ± 1.5 ab
ASR53	11.5 ± 0.4 b	6.7 ± 0.1 g	877 ± 27.5 g	114.2 ± 5.5 d
ASR54	10.2 ± 0.4 ab	5.7 ± 0.7 efg	618 ± 42.0 abcd	99.9 ± 12 bcd
ASR56	10.5 ± 1.4 ab	4.7 ± 0.7 cd	701 ± 22.6 cdef	91.0 ± 5.7abc
ASR58	9.8 ± 1.4 ab	4.0 ± 0.3 bc	720 ± 30.0 ef	90.0 ± 9.3 ab
ASR65	10.6 ± 1.0 ab	3.5 ± 0.7 ab	671 ± 35.0 bcdef	92.2 ± 2.5 abc
ASR67	9.9 ± 0.9 ab	3.4 ± 0.3 ab	651 ± 47.0 bcde	91.2 ± 2.5abc
ASR75	8.2 ± 0.5 a	5.4 ± 0.8 def	590 ± 17.5 ab	72.5 ± 9.4 a
ASR76	10.3 ± 1.1 ab	4.8 ± 0.1 cde	729 ± 27.0 ef	101.4 ± 11.1 bcd
Maconzeb	11.1 ± 0.8 b	4.0 ± 0.3 bc	603 ± 28.0 abc	101.7 ± 8.6 bcd

The different letters indicate the data were significantly different as analyzed by Tukey test ($p \leq 0.05$)

2.7 to 6.7 cm, fresh weight from 522 to 877 mg · plant^{-1}, and dry weight from 83.2 to 114.2 mg · plant^{-1}.

Molecular identification of selected actinomycetes

The isolate was identified based on 16S rRNA partial gene analysis. The DNA target of the most potential isolates was aligned to the representative sequence from the database using the BLASTN program. Phylogenetic analysis of the 16S rRNA sequence of the isolate ASR53 showed that ASR53 was similar to genus *Streptomyces* (Fig. 4). Isolate ASR53 showed high similarity (98.8%) to *S. violaceorubidus* LMG 20319 (Table 4).

Bioactive compound of *Streptomyces* sp. ASR53 crude extract

Gas chromatography-mass spectrometry (GC-MS) analysis identified 10 compounds present in the methanolic extract of isolate ASR53 (Table 5). Several dominant compounds were identified as antifungal bioactive compounds such as: dodecanoic acid, 1,2,3-propanetriyl ester; pyrrolo [1,2-a]pyrazine-1,4-dione; 1,4-diaza-2,5-dioxo-3-isobutyl bicyclo [4.3.0] nonane; 9-octadecenamide (Z); 3-benzyl--1,4-diaza-2,5-dioxobiocyclo [4.3.0] nonane, and lauric acid.

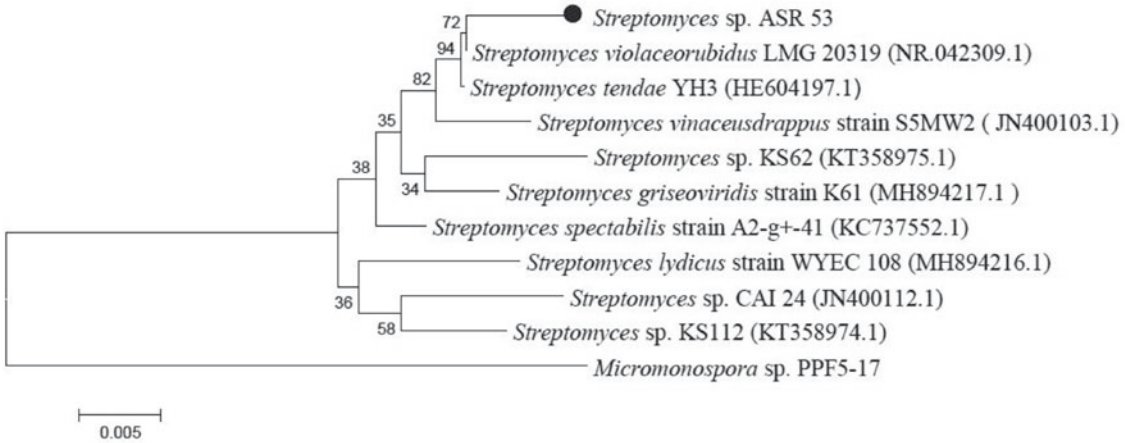

Fig. 4. Neighbor-joining phylogenetic tree based on the partial gene of 16S rRNA gene sequence of isolate ASR53 showing the phylogenetic relationship between strain *Streptomyces* sp. and some other taxa. Black square showed rhizosphere actinomycetes used in this study

Table 4. 16S rRNA genes sequence alignment of selected rhizospheric actinomycete isolates using BLASTN program

Isolate	Homolog species (GenBank)	Query cover [%]	E-value	Identity [%]	Accession number
ASR53	*Streptomyces violaceorubidus* (LMG 20319)	100	0.0	98.8	NR. 042309.1
	Streptomyces tendae (NBRC 12822)	100	0.0	98.7	NR. 112290.1
	Streptomyces tritoleran (DAS 165)	100	0.0	98.5	NR. 043745.1

Table 5. Chemical composition of the actinomycete crude extract of *Streptomyces* sp. ASR53

No.	Compound	Peak area [%]	Retention time	Similarity [%]	Bioactivity	Reference
1	Dodecanoic acid, 1,2,3-propanetriyl ester	44.97	33.64	76	antifungal antibacterial	Walters *et al.* 2003, Anzaku *et al.* 2017
2	Pyrrolo [1,2-a] pyrazine--1,4-dione	9.35	10.39	80	antifungal antibacterial antioxidant	Awla *et al.* 2016, Kiran *et al.* 2018, Ser *et al.* 2015
3	Colchiceine	5.18	15.35	38	therapeutic agent	Leung *et al.* 2015
4	Glyceril dodecanoate	4.37	15.56	60	unknown	
5	9-Octadecenamide (Z)	3.47	12.06	98	antifungal and antibacterial	Mohammed *et al.* 2016
6	1,4-Diaza-2,5-dioxo-3-isobutyl bicyclo [4.3.0] nonane	3.29	10.20	72	antifungal	Hanif *et al.* 2016
7	4-Dibenzofuramine	3.19	15.73	43	antibacterial	Shou *et al.* 2012
8	3-Benzyl-1,4-diaza-2,5-dioxobiocyclo [4.3.0] nonane	2.67	12.33	97	antifungal	Hanif *et al.* 2016
9	6-Chloro-2-(2-hydroxyethylamino)-	2.01	15.19	47	unknown	
10	Lauric acid	1.83	15.01	47	antifungal antibacterial	Walters *et al.* 2003, Anzaku *et al.* 2017

Discussion

Exploration of a new biocontrol agent which is not only capable of reducing the soil-borne disease particularly against *R. solani,* but also of supporting the initial growth of the plant is necessary. In our previous study, the capability of 26 soybean rhizosphere actinomycete isolates to enhance the growth of soybean germination was conducted as *in vitro* analysis. The results indicated that the tested isolates acted as growth promoters, through producing IAA, solubilizing phosphate, increasing shoot and root lengths and the number of lateral roots *in vitro* (data not shown). These isolates are expected to be developed as a biofertilizer that improves plant growth and suppresses plant disease.

Of 26 isolates tested, 10 (31%) actinomycete isolates were found to have stronger antifungal activity (≥40%) against *R. solani* than the other eight isolates which had lower antifungal activities (ranging from 18.9 to 64.8%). Isolate ASR53 showed the highest inhibition against *R. solani* by 64.8%. The different antifungal activities were possibly influenced by the different types of bioactive compounds produced by the isolates. These results indicate that there is high chemical diversity of antifungal compounds from the rhizosphere actinomycetes. The benefit of antifungal compounds in controlling pathogenic fungi is that these compounds will diffuse in the soil and consequently do not need direct contact with the pathogen (Goudjal *et al.* 2016).

One of the important requirements for biocontrol agents of soybean diseases is not being antagonistic towards nodule bacteria especially *B. japonicum*. A compatibility assay towards *B. japonicum* strain BJ11 showed that all the tested isolates could grow synergistically with *B. japonicum* strain BJ11 as indicated by the lack of a clear zone formation around actinomycete colonies. The commensal interaction between actinomycetes and *B. japonicum* could support plant growth by increasing root nodulation frequency, leading to larger sized nodules, which improves bacteroids'

vigor within the nodules, consequently iron and other nutrients are increased (Tokala *et al.* 2002).

Ten isolates with antifungal activity not only showed activity *in vitro* but also *in planta*. In the greenhouse assay 10 isolates showed various disease suppression and disease incidence in soybean plants infested with *R. solani*. The highest disease suppression was seen by isolate ASR53 both in sterile (68.3%) and in non-sterile soil (91.1%). These results suggest that isolate ASR53 consistently reduced disease with or without interaction with indigenous soil microbes. Other isolates, ASR67 and ASR58 could inhibit disease symptoms under different soil conditions. Isolate ASR67 suppressed disease more effectively in sterile soil than in non-sterile soil. Isolate ASR58 was more effective in non-sterilized soil than in sterilized soil. These results demonstrated that the nutrient content and the presence of indigenous microbes probably influenced disease suppression activity from actinomycete isolates. Mahmood *et al.* (2014) felt that sterilized soil may lose some nutrients, particularly amino acids, organic compounds and soil microbes by high temperature. This study suggested that disease suppression from the 10 actinomycetes isolates could be affected not only by the existence of other indigenous antagonistic microbes which synergestically work with antagonist isolates, but also the content of soil nutrients. Moreover, seeds, coated with mancozeb as a chemical fungicide, also significantly decreased ($p < 0.05$) by over 78.6% in non-sterilized soil and 63% in sterilized soil. Nevertheless, regarding their efficacy, the use of synthetic fungicides needs to be considered due to their effects on resistance and contamination of the environment.

Biocontrol activities of actinomycetes are often associated with the promotion of plant growth. In our study, growth enhancement of soybean seedlings by the rhizosphere actinomycetes were evaluated. Isolate ASR53 showed a beneficial effect in promoting the growth of soybean seedlings and it significantly increased the root length, shoot length and dry weight of tomato seedlings compared to positive control, both in sterile and non-sterile soil. These findings are consistent with those shown by *Streptomyces* sp. strain C (Sadeghi *et al.* 2009) and *S. asterosporus* strain SNL2 (Goudjal *et al.* 2016), which significantly improved the growth of tomato seedlings.

Based on their antagonistic activity *in vitro*, selected rhizosphere actinomycetes were effective in reducing the incidence of *R. solani* damping-off disease in sterilized and non-sterilized soil. Their biocontrol activities might be caused by several mechanisms, such as: antibiosis, hyperparasitism, and cell wall degrading enzyme (Palaniyandi *et al.* 2016). As mentioned before, the selected isolates were shown to be plant growth promoters in the previous study which also established that they have a role in damping-off disease suppression. In addition, *Streptomyces* spp. also caused hyphal interaction including coiling, penetration, branching of the growing hyphae leading to granulation, coagulation of the cytoplasm, enlargement of the cytoplasmic vacuole, hyphal deformation and hyphal lysis (Huang *et al.* 2012).

In this study, isolate ASR53 showed the highest antifungal activity that was consistent in both *in vitro* and *in planta*. Biocontrol activity of ASR53 was possibly caused by some bioactive compounds produced by this isolate. Based on GC-MS analysis, crude extract derived from ASR53 contained 10 dominant compounds including dodecanoic acid, 1,2,3-propanetriyl ester; pyrrolo [1,2-a]pyrazine-1,4-dione; colchiceine; glyceril dodecanoate; 9-octadecenamide (Z); 1,4-diaza-2,5-dioxo-3-isobutylbicyclo[4.3.0]nonane; 4-dibenzofuramine; 3-benzyl-1,4-diaza-2,5-dioxobiocyclo[4.3.0]nonane; 6-chloro-2-(2-hydroxyethylamino)- and lauric acid. Some of them have antifungal activity against *R. solani* and *Phytium ultimum* (Walters *et al.* 2003), *Pyricularia oryzae* (Awla *et al.* 2016), and *Fusarium oxysporum* (Hanif *et al.* 2016). The bioactivities of two other major compounds including glyceril dodecanoate and 6-chloro-2-(2-hydroxyethylamino) have not been studied yet. These compounds need to be further investigated. Therefore, we suggest that these compounds might have a significant role in antifungal activities of isolate ASR53. Based on previous research the hypothesis that ASR53 is the most promising actinomycete isolate for managing *R. solani* damping-off disease was also supported by other characteristics such as the ability to produce siderophore, chitinase, and HCN.

Based on 16S rRNA, the most potential isolate, ASR53 has been identified as *S. violaceorubidus* (Fig. 2). Several studies reported that *Streptomyces* possess biocontrol properties in soil-borne diseases. *Streptomyces* spp. also provide better efficacy in controlling *R. solani* damping-off sugar beet compared to the synthetic fungicide Vitavax (Sadeghi *et al.* 2009). *Streptomyces* spp. isolated from herbal vermicompost are found to have suppression disease capacity against *Fusarium oxysporum* (FOC) in the field (Gopalakrishnan *et al.* 2011). Moreover, registered commercial products from *Streptomyces* such as Mycostop® (*Streptomyces griseoviridis* strain K61) and Actinovate® (*Streptomyces lycidus* strain WYEC108), have also been applied to reduce damping-off disease (Shimizu 2011).

Conclusions

The study revealed that actinomycetes from the soybean rhizosphere can potentially be used to develop biocontrol agents that reduce the growth of *R. solani*, both

in vitro and *in planta*. In the greenhouse experiment, isolate ASR53 showed the highest disease suppression to *R. solani* damping-off disease in the soybean plant. Seed coating treatment with isolate ASR53 in sterilized and non-sterilized soil showed better growth than the pathogenic control and synthetic fungicide treatment. Chemical compound analysis from the crude extract of ASR53 by GC-MS resulted in 10 chemical constituents, with dodecanoic acid, 1,2,3-propanetryil ester as the major compound. Based on these results, it is obvious that *Streptomyces* sp. ASR53 obtained from the soybean rhizosphere is a promising candidate for the development of a biocontrol agent. This would have an important role in the establishment of early plant growth and defense toward the pathogen.

Acknowledgements

This work was partially supported by the Ministry of Research, Technology, and Higher Education of Indonesia through "Penelitian Dasar Unggulan Perguruan Tinggi (PD-UPT)" 2018–2019 and Dissertation Research Grant from LPPM Sebelas Maret University 2017.

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