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Diversity of biocontrol agents, isolated from several sources, inhibitory to several fungal plant pathogens

YAN RAMONA[1,2,♥], IDA BAGUS GEDE DARMAYASA[1], ANAK AGUNG NGURAH NARA KUSUMA[3], MARTIN A. LINE[4]

[1] Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Udayana. Jl.Raya Kampus Unud No. 9, Jimbaran, Badung 80361, Bali, Indonesia. Tel.: +62-361-701954, ♥email: yan_ramona@unud.ac.id
[2]Integrated Laboratory for Biosciences and Biotechnology, Universitas Udayana. Jl. Raya Kampus Unud No. 9, Jimbaran, Badung 80361, Bali, Indonesia
[3]STIKES Bina Usada Bali. Jl. Raya Padang Luwih, Tegal Jaya, Dalung, Badung 80361, Bali, Indonesia
[4]School of Agricultural Science, Hobart Campus, Tasmania University. Churchill Ave, Hobart TAS 7005, Australia

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Abstract. *Ramona Y, Darmayasa IBG, Kusuma AANN, Line MA. 2020. Diversity of biocontrol agents, isolated from several sources, inhibitory to several fungal plant pathogens. Biodiversitas 22: 298-303.* This study investigated the inhibitory potential of diversity of antagonist bacteria residing in the rhizosphere zone and mature compost to counter fungal plant pathogens. Soils collected from rhizosphere of lettuce farms in Bali-Indonesia and Tasmania-Australia, mature compost, commercial biocontrol (Dipel®), and laboratory contaminants with significant inhibition against tested fungal pathogens were used as sources of antagonist bacteria. These antagonists were isolated by applying dilution and spread method on trypticase soya agar (TSA) or potato dextrose agar (PDA), and their ability to inhibit *Sclerotinia minor*, *Sclerotinia sclerotiorum, Fusarium* spp., and *Rhizoctonia solani* was assessed in dual culture assays. The results showed that 67 out of more than 100 isolates had antagonistic activity *in vitro* against at least one of tested fungal pathogens. In the preliminary identification, *Bacillus* spp. or *Pseudomonas* spp. were found to be pre-dominant isolates. Following screening studies in a non-replicated glasshouse experiment against *S. minor* and *S. sclerotiorum*, 8 of the most promising isolates were further identified using molecular methods based on their 16s rDNA sequences aligned with those deposited at the GeneBank. These 8 isolates were identified as *Pseudomonas corrugata, Bacillus megaterium, Bacillus polymyxa, Bacillus mojavensis, Bacillus pumilus, Bacillus thuringiensis, Exiguobacterium acetylicum,* and *Chryseobacterium indologenes.*

Keywords: Antagonist, biological control, compost, diversity, plant pathogens, rhizosphere

INTRODUCTION

Isolation, screening, and identification are three first stages in the investigation of antagonist microbial diversity in soil samples, with a goal to develop biocontrol agents inhibitory to fungal plant pathogens. Soils, especially in the rhizosphere or rhizoplane of plant roots are commonly colonized by abundant candidates of biocontrol agents. In addition to soils, mature compost or decomposed plant materials are also often used as sources of biocontrol agent candidates. Until recently, root region of plants was still preferred as the main source of antagonists of plant pathogens (Suthar et al. 2016), although only small portion of those biocontrol candidates are culturable in vitro (Malleswari 2014).

In the last 3 decades, isolation of antagonists of plant pathogens has been intensively carried out globally with a view to develop more environmentally friendly solutions to reduce excessive application of chemical-based pesticides in farming practices. Chemicals have been used in many aspects of our life, including farming activities to control plant pathogens, and the negative side effects of such compounds in human life have been well recognized (Kim et al. 2016; Nicolopoulou-Stamati et al. 2016). Accumulation of such chemicals in soil has been reported to cause depletion of some beneficial soil microbiota, particularly plant growth-promoting rhizobacteria and mycorrhiza (Meena et al. 2020). Lucas et al. (2015) reported that induction of resistance mechanism in plant pathogens may also be due to excessive application of chemical pesticides in farming practices, and this, in turn, leads to an increased dose or invention of new pesticides to control the same pathogens (Fernandes et al. 2010).

The most acceptable strategy to isolate biocontrol agent candidates is to isolate them from the root or rhizosphere of the specific crop where they are intended to be applied to protect the plant, as they are already closely associated with and well adapted to such environmental conditions (Larkin and Fravel 1998). Ciancio et al. (2016) intensively reviewed several improved screening methods to identify likely antagonist candidates. Screening methods developed in the isolation of antagonist candidates should consider the relatedness of those antagonist candidates with their habitats. The conditions of isolation should be adjusted as close as possible with those where such agents are to be applied (Ciancio et al. 2016).

In the present study, the antagonists were isolated from rhizosphere of lettuce plants in Bedugul village of Bali, Indonesia and in Tasmania, Australia because, at the time of sample collection, farms in these areas were planted with lettuce plants against whose pathogens the antagonists are targeted. These antagonist candidates were targeted for use to protect such lettuce plants from attack by their pathogens (*Sclerotinia minor* and/or *Sclerotinia sclerotiorum*).

Another source of our antagonists was mature compost purchased from a nursery in Denpasar, Bali-Indonesia as well as in Tasmania in Australia. The rationale to target mature compost was due to a report of Gehan et al. (2018) where it was suggested that mature composts (including vermicompost) are also abundantly colonized by antagonists of fungal pathogens. Isolates with ability to significantly inhibit selected plant pathogens *in vitro* were screened in a non-replicated glasshouse pot trial before being further identified using molecular methods based on nucleotide sequence of the isolates. Molecular identification of most potential antagonists, isolated from areas mentioned above, will advance the understanding of the diversity of bacterial antagonists inhibitory to several plant pathogens.

Based on the above rationale, the main objective of this present study was to investigate the diversity of antagonist isolates residing in various sources, particularly in rhizosphere zone of lettuce plants, with the goal to develop biocontrol agents antagonistic against fungal pathogens, such as *S. minor*, *S. sclerotiorum*, *Fusarium* spp. and *Rhizoctonia solani*. Following the isolation, molecular identification based on 16s rDNA was carried out. The results of this research provide the foundation to develop and advance novel environmentally friendly methods of plant-pathogen control in organic farming practices, so that use of chemical-based fungicides in farming practices can be significantly reduced or even eliminated.

MATERIALS AND METHODS

Isolation of potential antagonists

Soils collected from lettuce farms in Bali-Indonesia and in Tasmania-Australia were used as the main source of bacterial antagonists. Bacterial antagonists were also obtained from the stock culture collections of the School of Biology, Udayana University as well as from the Environmental Microbiology Laboratory, School of Agricultural Science Tasmania University, Australia. Serial dilution and spread plate method as specified in Wulansari et al. (2015) was applied in the isolation and purification of these biocontrol agents.

Fungal pathogens

Fungal pathogens (*Sclerotinia minor*, *S. sclerotiorum*, *Fusarium* sp., and *Rhizoctonia solani*) were obtained from stock culture collection of Laboratory of Environmental Microbiology, Tasmania University or Laboratory of Microbiology, School of Biology, Udayana University. For regular use, these pathogens were sub-cultured on fresh PDA plates. For long-term storage, they were placed in sterile distilled water and stored at 4ºC.

Dual culture assay for screening of potential biocontrol agents

Dual culture assays were conducted in vitro by challenging antagonists with tested pathogens on plates of PDA or TSA according to procedures specified in Wulansari et al. (2015).

Screening of potential antagonists in a non-replicated pot trial

The efficacy of biocontrol agents to protect lettuce seedlings from attack by either *S. minor* or *S sclerotiorum* in a non-replicated glasshouse trial was conducted by immersing the plant roots in suspension of 48 hour-old bacterial antagonists. This trial was aimed at selecting the most effective number of antagonist isolates to be used in further studies. The bacterial cell density in saline solution was approximately 10^8 cells/ml, and this was determined using a spectrophotometer at OD reading of 540 nm. Inoculation of pots after 1 week of acclimatization was done following the method as specified in Wulansari et al. (2015), and all pots were maintained in the glasshouse for up to 8 weeks until disease incidence appeared. The negative and positive controls of this trial were un-inoculated pots and those inoculated with pathogen only, respectively.

16s rDNA sequencing analysis of potential antagonist isolates

For molecular identification, the sequence of 16S rDNA of some potential bacterial antagonists was determined and compared with their counterparts in a clone library of known bacteria (http://www.ncbi.nlm.nih.gov). The detailed procedures for the molecular identification of the most potential antagonists included extraction of DNA, Prep-A-gene purification, Polymerase chain reaction using the HotStart Mastermix PCR kit, Qiagen, Purification of PCR product, and Sequencing reactions following the method specified in Ramona et al. (2015). Sequencing of the PCR product was conducted at the Molecular Biology Unit, School of Biomedical and Molecular Science, Griffith University, Queensland-Australia.

Data analysis

Data obtained in this research were descriptively analyzed. Statistical analysis was not conducted in this study as our rapid screening for potential antagonist isolates only applied a non replicated pot trial. This method however was found to be effective to screen most potential candidates of biocontrol agents among hundreds of isolates.

RESULTS AND DISCUSSION

Soils in the root rhizosphere are particularly the most suitable habitat for abundant bacteria antagonistic against fungal pathogens *Sclerotinia minor* or *Sclerotinia sclerotiorum*. Shelby et al. (2016) reviewed that these bacterial antagonists play a significant mutualistic role with their plant hosts, although only small portion of those antagonists is culturable (Martines-Viveros et al. 2010). Our study demonstrated that soil contained high population of bacterial antagonists as we could isolate more than 100 isolates. Among those isolates, 20 showed significant inhibition zones (0.00 ± 0.0 to 9.7 ± 0.7 mm) to at least one tested plant pathogens (*S. minor*, *S. sclerotiorum*, *Fusarium* sp, or *R. solani*). They were further screened in a non-

replicated pot trial prior to molecular identification, and the results were reported elsewhere by Ramona (2003).

Rhizosphere zone appeared to be the most favorable habitat for most biocontrol agents as they are often in mutual association with plant roots. Therefore, soil from this zone was used as the main source of antagonist candidates, although most of them have been reported to be non-culturable *in vitro* on synthetic media. In our previous research, an antagonist *Bacillus mojavensis*, isolated as a laboratory agar-plate contaminant also showed good results in the non-replicated glasshouse trial (Ramona 2003).

Dual culture using pathogen plus potential antagonist-based assay was found to be rapid, simple, and effective method to initially recognize antagonistic activity in broad screening of potential biocontrol agents. Until recently, research on biocontrol development still relied on this conventional method (Kunova et al. 2016). In dual culture assays conducted in our study indicated zone of inhibition and some growth abnormalities of *S. minor*, such as alteration of hyphal tip color, hyphal tips became swollen or lysed hyphal tips (Figure 1). Similar responses were reported on *Rhizoctonia solani* following dual culture assay with *Trichoderma virens* (Inayati et al. 2020).

Beyond simply being fungistatic, lethal effects of the active compounds released by the antagonists to their surroundings were also observed. This was indicated as some mycelial plugs of the pathogens taken from the edge of the inhibition zone in dual culture assay failed to recover following sub-culture onto fresh PDA. Formation of inhibition zone between antagonists and pathogens in dual culture assays was commonly reported to be due to antibiosis mechanism in addition to ferric siderophore activity (Ghorbanpour et al. 2018). Additionally, according to Anith et al. (2014) diameter of inhibition zone *in-vitro* may depend on types of medium used in the dual culture assays.

Our non-replicated glasshouse trial was found to be effective to limit numbers of antagonist isolates to be further studied (Table 1). In this trial, *S. minor* was found to be more aggressive than *S. sclerotiorum* in attacking lettuce seedlings/plant, as infection by *S. minor* appeared within days after inoculation rather than several weeks after inoculation. Isolates of 8 antagonists (UT1, SAJ6, PA, Bali C, and Bali G, RC antagonist, SBJ4, and a commercially available strain Dipel®) showed significantly higher protection (ranging from 25% to 50%), when compared to the control treatment were selected for further identification using molecular methodology based on sequencing of their 16s rDNA.

Table 1. Non-replicated trial in a glasshouse scale experiment to assess the efficacy of selected antagonists to prevent lettuce seedling infection by *Sclerotinia minor* or *S. sclerotiorum*

Treatments[1]	% of healthy plants after *S. minor* Inoculation (%)			% of healthy plants after *S. sclerotiorum* inoculation (%)		
	Days after pathogen inoculation			Weeks after pathogen inoculation		
	4	5	6	2	4	8
C7 (TSA)	25	0	0	100	50	0
C8 (TSA)	50	0	0	75	50	25
C9 (TSA)	75	25	0	100	25	0
UT1 (PDA)	75	25	25	75	25	25
UT4 (TSA)	25	25	0	100	0	0
RC ant. (TSA)	50	0	0	100	75	50
3A (TSA)	50	0	0	50	50	25
SAJ1 (TSA)	50	0	0	50	25	25
SAJ2 (TSA)	25	0	0	75	50	25
SAJ3 (TSA)	50	0	0	50	0	0
SAJ5 (TSA)	50	0	0	25	0	0
SAJ6 (TSA/PDA)	100	75	50	75	50	25
SAJ9 (TSA)	0	0	0	75	50	0
SBJ4 (TSA)	50	25	0	75	50	25
RW1 (TSA)	50	25	0	50	25	0
PA (PDA)	75	50	25	100	50	50
Bali C (TSA)	25	25	25	75	25	25
Bali E (TSA)	25	0	0	50	25	0
Bali G (TSA)	50	50	25	100	50	50
Dipel (TSA)	25	25	0	100	50	25
A0B1*	0	0	0	25	0	0
A0B0**	100	100	100	100	100	100

Note: [1]Each pot was sown with 4 lettuce seedlings. *Pots inoculated with either *S. minor* or *S. sclerotiorum* only served as control treatments. **Nil control (neither pathogen nor antagonist was inoculated)

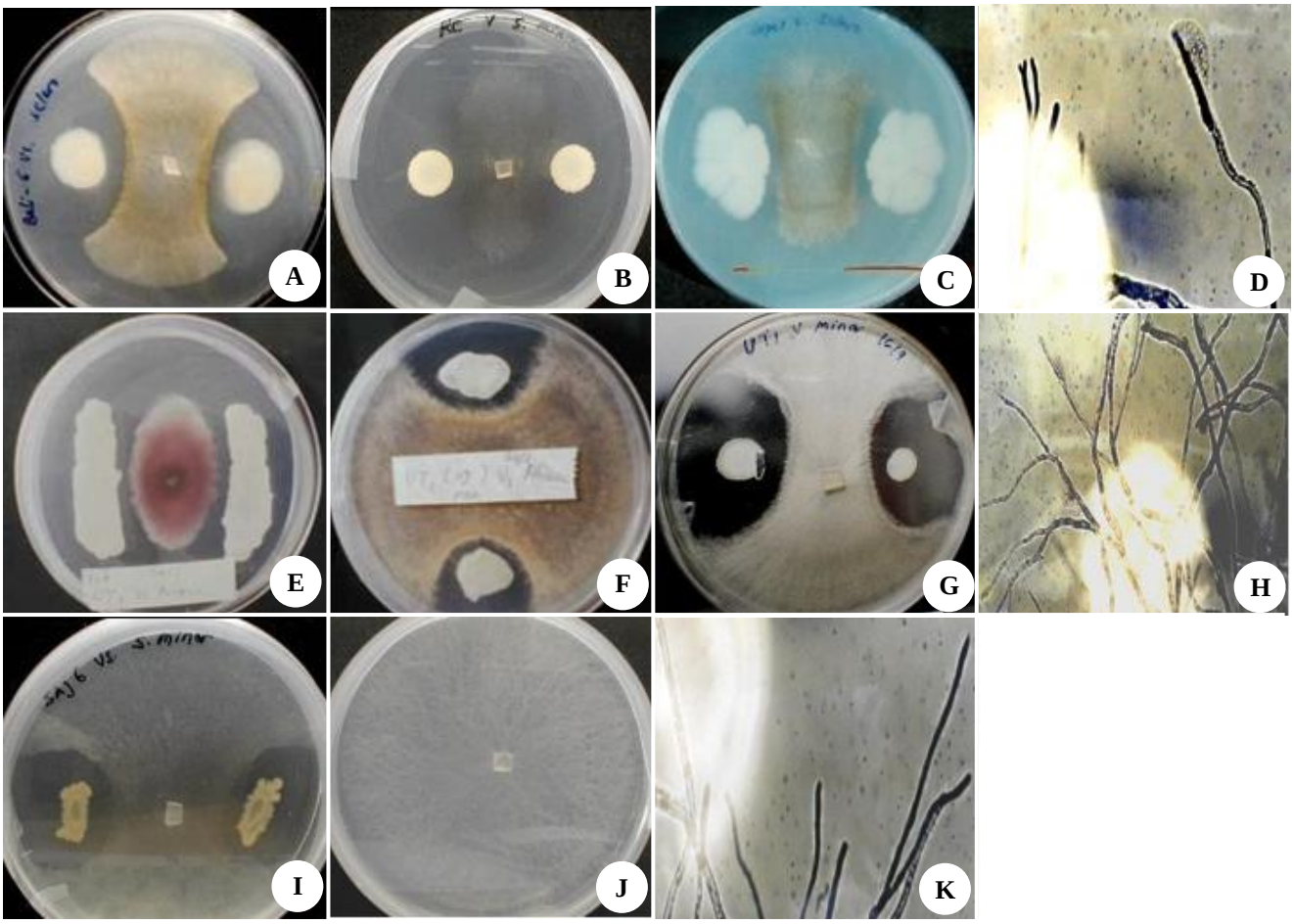

Figure 1. Inhibition zones in dual culture assays produced by some bacterial antagonists on fungal plant pathogens. A and C are inhibition zones of *Lysobacter antibioticus* and *Bacillus thuringiensis* respectively on *S. sclerotiorum*; B, F, and G are inhibition zones of *Bacillus pumilus* and *Bacillus polymyxa,* and *Pseudomonas corrugata* respectively on *S. minor*; D and E are inhibition zones of *Bacillus polymyxa* on *Fusarium* sp. and *R. solani*, respectively; H is normal growth of *S. minor*; and I is microscopic normal growth of *S. minor* hyphal tips on potato dextrose agar. Colour alteration, swollen hyphal tips, and lysed of *S. minor* mycelial tips, indicating growth abnormalities, are shown by arrowheads (A, J, and K, respectively)

Preliminary identification which included several biochemical reaction tests (Gram stain, spore stain, motility, flagella stain, oxidative/fermentative capability, oxidase activity, catalase activity, casein hydrolysis capability, starch hydrolysis capability, urease activity, methyl red and Voges-Proskauer test, citrate utilization, indole formation, UV fluorescence, levan production, and gelatin hydrolysis capability) was reported in our previous study (Ramona 2003). Most isolates belong to genera of *Bacillus* or *Pseudomonas* spp. Other isolate cultures found to be less frequent, such as *Acinetobacter, Flavobacterium, Moraxella, Alcaligenes, Chromobacterium, Erwinia, Brevibacterium*, or *Proteus* species were also identified.

Molecular identification conducted in this current study on the 8 most potential isolates (SAJ6, PA, RC antagonist, Bali C, Bali G, Bali J, SBJ4, and a bacterium isolated from commercially available biocontrol product Dipel®) showed that they were closely related to *Pseudomonas corrugata*, *Bacillus mojavensis, B. pumilus*, *Exigoubacterium acetylicum*, *Lysobacter antibioticus*, *Chryseobacterium indologenes*, *B. megaterium*, and *B. thuringiensis* (Dipel), respectively (Figure 2). This molecular identification provided more definitive identity of these isolates following alignment of their 16s rDNA sequences with those deposited at the Gen-Bank Nucleotide Database Library using GAPPED BLAST on-line searches (http://www.ncbi.nml.nih.gov/blast/blast.cgi) (Mauti et al. 2013; da Costa Capizzani et al. 2018). *Bacillus thuringiensis, B. pumilus, B. mojavensis, B. megaterium*, and *Pseudomonas corrugata* appeared to be the same species as those frequently reported as biocontrol agents in the literature (e.g. by Wang et al. 2020; Heidarzadeh and Baghaee-Ravari 2015; Ntushelo et al. 2019; Zheng and Sinclare 2000; and Catara 2007, respectively). Rare reports on the potential use of *Lysobacter antibioticus*, *Exiguobacterium acetylicum,* and *Chryseobacterium indologenes* as biocontrol agents could be found.

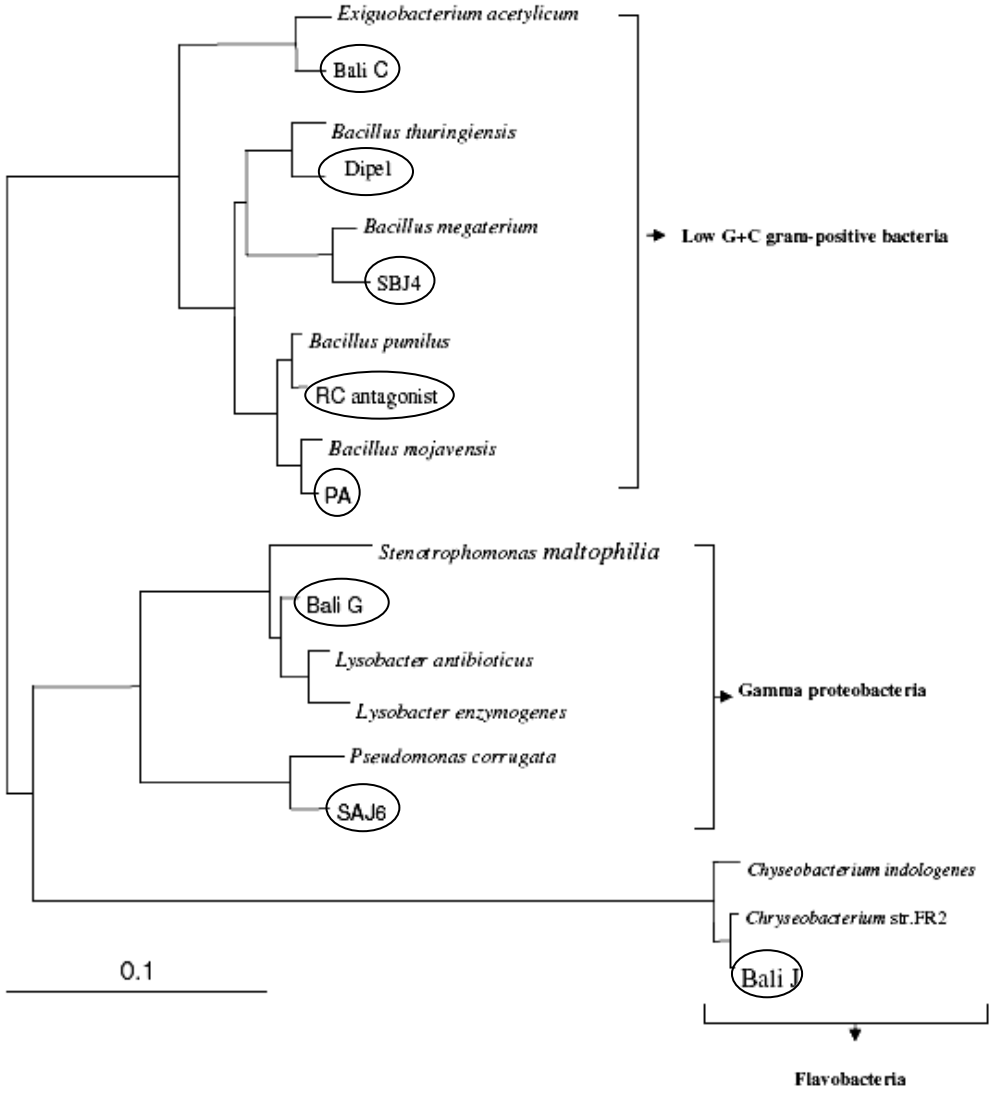

Figure 2. The relatedness of selected antagonists with those deposited in the GeneBank. The bar indicates 0.1% rDNA difference in relatedness

This study clearly demonstrated that high diversity of antagonist bacteria could be found in the lettuce rhizosphere zone, although only small portions were culturable *in vitro*. Besides that, only a limited number of those isolates were found effective to control plant pathogens *in vitro* or in glasshouse scale experiments. Among more than 100 isolates obtained, only 8 isolates were found to be consistently inhibitory to *S. minor* and *S. sclerotiorum*, and they were closely related genetically to *Pseudomonas corrugata, Bacillus megaterium, Bacillus polymyxa, Bacillus mojavensis, Bacillus pumilus, Bacillus thuringiensis, Exiguobacterium acetylicum, Chryseobacterium indologenes* following alignment of their 16s rDNA with those deposited in the Gen-Bank Nucleotide Database Library. The results of this study has significant implications for the future development of biocontrol methods and solutions to reduce application of chemical-based fungicides in organic farming practices. Inhibitory compounds (anti-fungal compounds) produced by these identified antagonists need to be extracted in future study, so that the mechanisms by which such antagonists inhibited the tested pathogens can be elucidated. This will make it possible to produce large scale biological-based compounds inhibitory to plant pathogens in the future allowing more sustainable biological methods for controlling fungal plant pathogens.

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