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ISOLATION AND CHARACTERIZATION OF RHIZOBACTERIA FOR BIOLOGICAL CONTROL OF ROOT-KNOT NEMATODES IN INDONESIA

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ABSTRACT

Four rhizobacteria isolated from a potato field were assesed for their antagonistic potential against root-knot nematode (*Meloidogyne* spp.) affecting potato crops *in vitro*. Pure cultures of rhizobacteria were characterized based on their morphology, physiology, and biochemistry, as well as biofertilizer and biopesticide activities. An *in vitro* screening of bacterial strains against root-knot nematodes was conducted in a 25-microwell plate for 4 hours after nematode application. Four potential antagonistic bacteria were able to kill *Meloidogyne* spp. and were selected from the *in vitro* assay. These four bacteria were identified as *Serratia marcescens*, *Serratia* sp., *Vibrio* sp., and *Aeromonas* sp. Percentage nematode mortality after placing second stage nematode juveniles 2 (J2) in sterile distilled water for 12 hours ranged from 98.20 to 99.87%, with *Aeromonas* sp. showing the highest percentage nematode mortality (99.87%). Additionally, all rhizobacterial strains were positive for the production of gelatinase, protease, and chitinase enzymes, and were considered as biopesticides. This experiment indicated that all selected bacterial strains associated with potato have potential as environmental-friendly biocontrol agents against root-knot nematode in potato. For any future studies, bacterial efficacy in pots and field trials should also be tested.

Key words: *Meloidogyne* spp., *Serratia* sp., *Vibrio* sp., *Aeromonas* sp.

INTRODUCTION

Plant parasitic nematodes, particularly sedentary endoparasitic nematodes, are dominant pests of a wide variety of crops, causing more than 100 billion dollars in economic loss worldwide. Root-knot nematodes (*Meloidogyne* spp.) are the most economically-important worldwide, having impact both on crop yield and quality (Sasser and Freckman 1987). They affect approximately 2,000 plant species, with most cultivated crops being attacked by at least one root-knot nematode species (Bird et al. 2009). Root-knot nematodes are more difficult to control than other pathogens because they are soil-borne inhabitants, always attacking underground plant parts (Stirling 1991). Root-knot nematodes are commonly managed by chemical nematicides, including soil fumigant and non-fumigant nematicides. However, some developed countries have begun to withdraw chemical nematicides due to public health and environmental safety concerns (Schneider et al. 2003). The use of chemicals causes environmental damage and are toxic to humans, animals, and plants. As a consequence, new alternatives of nematode control that are more environmental-friendly have become increasingly important in order to avoid continuous yield loss caused by root-knot nematodes. Among these new alternatives, biological control using antagonistic microorganisms to suppress diseases have been explored (Pal and Gardener 2006).

Root-knot nematodes as soil inhabitants are subjected to infections by bacteria and fungi, thus, the use of soil microorganisms as antagonistic microorganisms is a good possibility (Jatala 1986, Mankau 1980). Numerous varieties of microorganisms and natural enemies antagonistic to soil nematodes are usually found in "nematode-suppressive" soils, wherein pathogens infect plants below economic thresholds (Park et al. 2014). Suppressive soils are soils that allow the pathogen to establish but not cause disease, or allow initial pathogen establishment and severe disease, followed by diminished disease severity, with continued growth of the host (Baker and Cook 1974). Of these pathogens, bacteria are considered the most abundant soil microorganism with some of them proved to be great potential biological control agents against nematodes, such as members of the genera *Pasteuria*, *Pseudomonas*, and *Bacillus* (Emmert and Handelsman 1999, Meyer 2003, Siddiqui and Mahmood 1999, Seenivasan 2018).

In Indonesia, root-knot nematodes are increasingly important major pests, affecting a wide variety of hosts, particularly vegetables. Several researchers have tested the use of alternative control methods, including the use of biofumigants (Edward and Ploeg 2014, Laquale et al. 2015, Salem and Mahdy 2015, Sari et al. 2018), as well as biocontrols applying antagonistic microorganisms (Mulyadi et al. 1995, Munif et al. 2013, Munif et al. 2015, Pradana et al. 2016, Utari et al. 2018). However, the use of antagonistic microorganisms against root-knot nematodes affecting potato in North Sumatra, Indonesia has not been explored thoroughly. Only several soil fungi assosiated with the potato rhizosphere were tested against *Meloidogyne* spp. affecting potatoes during a field-trial in Karo district, North Sumatra. Results revealed that *Trichoderma* spp. could significantly reduce nematode galls, while *Mucor* sp. improved potato growth (Utari et al. 2018). Antagonistic fungi (*T. harzianum, T. koningii* and *T. viride)* exhibited good potential in controlling *Meloidogyne* spp. *in vitro* wherein the hatching of nematode eggs was inhibited by up to 47.47% (Santoso et al. 2014). On the other hand, antagonistic bacteria *Pseudomonas fluorescens* and *Bacillus subtilis* killed *Meloidogyne javanica* by 42.85% and 29.28%, respectively (Yus et al. 2014). In this study bacteria isolated from potato rhizophere were screened through an *in vitro* assay against root-knot nematode (*Meloidogyne* spp.).

MATERIALS AND METHODS

Isolation and screening of potential rhizobacteria as biocontrol agents

Bacterial strains were isolated from root-knot nematode suppressive soil collected from the rhizosphere of potatoes through dilution-plate technique (Danzinger et al. 1990). One hundred grams of soil samples from four villages, including Juma Genting, Gajah, Raya and Aji Julu in Karo district, North Sumatra, Indonesia, were collected using purposive random sampling of ten points of both rhizosphere and soil that were compiled together. Soil samples were analyzed in the Laboratory of Plant Diseases, Faculty of Agriculture, Universitas Sumatera Utara, Indonesia. Ten grams soil from each sample was mixed aseptically with one liter of sterilized distilled water containing sodium chloride (Merck) and 0.002% Tween 80 (Merck). One ml of each suspension from both 10^4 and 10^6 serial dilutions were poured aseptically on solidified nutrient agar medium (peptic digest of animal tissue 5 g, sodium chloride 5 g, beef extract 1.5 g, yeast extract 1.5 g, agar (Difco) 15 g, distilled water 1L; with pH adjusted to 7.4) (Himedia) and then incubated at 28° C for two days. The bacteria isolated were cultured in solidified nutrient agar medium and then stored at 4 °C for experimental use.

Characterization and identification of rhizobacteria

Biochemical tests

Selected bacterial strains were characterized and identified according to Bergey's Manual of Determinative Bacteriology (Holt et al. 1994). The identity of isolated bacteria was confirmed through macroscopic (cell shape, cell arrangememt, cell size, colony shape, and colony colour), and microscopic (gram staining, cell shape, cell arrangement, and endospore) observations (Microscope Olympus, 1000x). The presence of bacterial growth on solidified nutrient agar at 30 and 37 °C was

investigated. Biochemical assays, including oxidase test (Kovacs 1956), catalase test (He et al. 1983), oxidative/fermentative Hugh and Leifson method (Schaad et al. 2001), indole test (MacFaddin 1980), methyl red (McDevitt 2009), Voges-Phoskaeuer (McDevitt 2009), citrate utilization (Simmons (1926) in Collins et al. 1989), were also conducted. Biofertilizer assays, such as the determination of phosphate solubility (Paul and Sinha 2013) and indole acetic acid/tryphtophanase (Fischer et al. 2007), were performed. For the biopesticide assay, the production of enzymes gelatinase (Frazier 1926), protease (Abebe et al. 2014), and chitinase (Frändberg and Schnuter 1997) were determined. Biofertilizer assay was conducted to determine the ability of tested bacterial strains to act as a plant growth enhancer.

Nematode isolation

Root-knot nematodes were collected from potato roots and washed under running water. Nematodes were isolated from egg-mass from galls. The nematode eggs were incubated in a microwell plate containing 3 ml of sterile distilled water for 16 hours at room temperature.

***Screening rhizobacterial strains against Meloidogyne spp.* in vitro**

Four bacterial strains were screened for their antagonistic activities against *Meloidogyne* spp. in microwell plate (25 wells). Heavy cell suspension for each bacterial strains was prepared at rate of 1 x 10^6 colony form unit (cfu)/ml in sterile distilled water using a spectrophotometer. Each well contained one hundred second stage juveniles (J2) of *Meloidogyne* spp. in 100 ml of sterile distilled water and 5 ml of bacterial suspension. *Meloidogyne* spp. J2 mortality was evaluated under different exposure times (1, 2, 3, and 4 hours). Nematode mortality was also examined upon confirming the mortality of J2 *Meloidogyne* spp. in sterile distilled water for 12 hours. The treatments were replicated three times.

Statistical analysis

Statistical analysis was performed using SPSS Statistics version 21.0.0 (International Business Machines Corporation, USA). For multiple-group comparison among the antagonistic bacterial strains and *Meloidogyne* spp. larvae, two-way ANOVA at $P = 0.05$ was performed. Ninety-five percent fiducial limits of experimental data were determined by probit analysis. All data were expressed as mean ± SD (standard deviation).

RESULTS AND DISCUSSION

Four bacterial strains, including K1 (*Serratia marcescens*), K2 (*Serratia* sp.), K3 (*Vibrio* sp.) and K4 (*Aeromonas* sp.), were present in the potato's rhizosphere in four villages of Karo District, North Sumatra, Indonesia (Fig. 1). The bacterial strains were grown on solidified nutrient agar (NA) and further identified through macroscopic and microscopic characterization (Fig. 2, Table 1).

Colonies from strains (*S. marcescens*) and *Serratia* sp. showed red colour on NA agar media (Himedia) while colonies from strains *Vibrio* sp. and*Aeromonas* sp. were cream white (Figure 1). Bacterial cell from the strains *S. marcescens*, *Serratia* sp., *Vibrio* sp. and *Aeromonas* sp. were rod-shaped and had a size of 1.4µm x 0.4 µm, 1.9µm x 0.8 µm, 2.3µm x 0.8 µm and 1.1µm x 0.8 µm, respectively (Figure 2). However, all of the four strains had similar shape, elevation, and margin, i.e. circular, convex, and entire, respectively (Table 1). Based on the morphological and biochemical characterization results in Table 1, the rhizosphere bacteria isolated from potatoes in Karo district, North Sumatra, were identified as follows: K1 - *Serratia marcescens*; K2 - *Serratia* sp.; K3 - *Vibrio* sp.; and K4 - *Aeromonas* sp.

Table 1. Characteristics of antagonistic bacterial strains.

Characteristics	Strains			
	K1 (*Serratia marcescens*)	**K2 (*Serratia* sp.)**	**K3 (*Vibrio* sp.)**	**K4 (*Aeromonas* sp.)**
Cell shape:				
Coccobacilli	-	-	-	+
Bacilli (Round End)	+	+	-	-
Curved	-	-	+	-
Cell arrangement:				
Pairs	+	+	-	-
Groups	-	-	+	+
Cell size:				
Length	1.4 µm	1.9 µm	2.3µm	1.1µm
Width	0.4 µm	0.8µm	0.8 µm	0.8 µm
Spores:				
Endospores	-	-	-	-
Terminal	-	-	-	-
Central	-	-	-	-
Subterminal	-	-	-	-
Exospore	-	-	-	-
Gram staining:				
	negative	negative	negative	negative
Colony shape:				
Circular	+	+	+	+

Characteristics	Strains			
	K1 (*Serratia marcescens*)	K2 (*Serratia* sp.)	K3 (*Vibrio* sp.)	K4 (*Aeromonas* sp.)
Irregular	-	-	-	-
Filamentous	-	-	-	-
Rhizoid	-	-	-	-
Flat	-	-	-	-
Umbonate	-	-	-	-
Caterifom	-	-	-	-
Entire	+	+	+	+
Undulate	-	-	-	-
Filliform	-	-	-	-
Curled	-	-	-	-
Lobate	-	-	-	-
Colony colour:				
Red	+	+	-	-
Oxidase (Kovac's sitochrome)	-	-	+	+
Glucose (OF Lefusion)	-/-	-/-	-/-	-/-
Catalase (H_2O_2)	+	+	+	+
Indole (Kovacs Indole)	-	-	+	+
Metyl Red	-	-	-	-
Voges-Phoskauer	-	-	-	-
Utilization of Citrate, (Simmon's)	+	+	+	+
Sulfur Indol Motility (Semi Solid) :				
Sulfur (H_2S)	-	-	+	+

Characteristics	Strains			
	K1 (*Serratia marcescens*)	**K2 (*Serratia* sp.)**	**K3 (*Vibrio* sp.)**	**K4 (*Aeromonas* sp.)**
Indole	-	-	-	-
Motility	+	-	+	+
Urenase	-	-	-	-
Triple Sugar Iron Agar:				
Glucose	-	-	-	-
Lactose	-	-	-	-
Methionine Fermentation	-	-	+	+
Utilization of Na+	-	-	+	-
Luminescent production	-	-	+	-
Growth at:				
30 °C	NA[1]	NA	-	NA
37 °C	NA	NA	-	NA
Fermentation:				
Glucose/ Gas	+/-	+/-	+/-	+/-
Lactose/Gas	-/-	-/-	+/-	+/-
Mannitol/ Gas	+/-	+/-	+/-	+/-
Maltose/ Gas	+/-	+/-	+/-	+/-
Enzyme Production:				
Chitinase	+	+	+	+
Protease	+	+	+	+
Lipase	+	+	+	+
Gelatine hydrolysis	+	+	+	-

[1] Not available

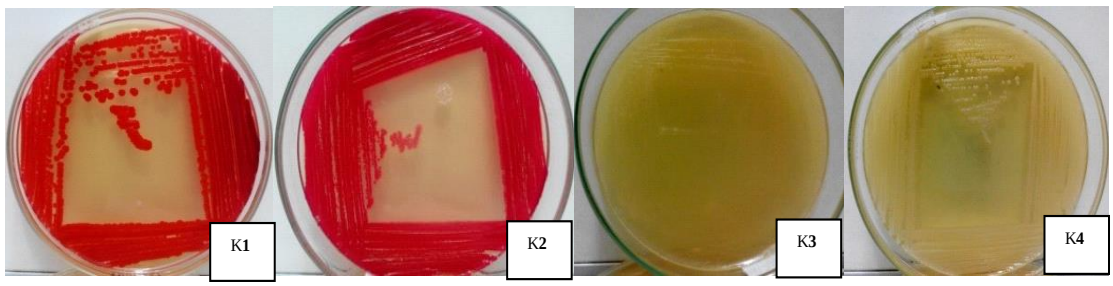


Fig. 1. Bacterial strains isolated from potatoes' rhizosphere in Karo District
K1: *Serratia marcescens*, K2: *Serratia* sp., K3: *Vibrio* sp., K4: *Aeromonas* sp.

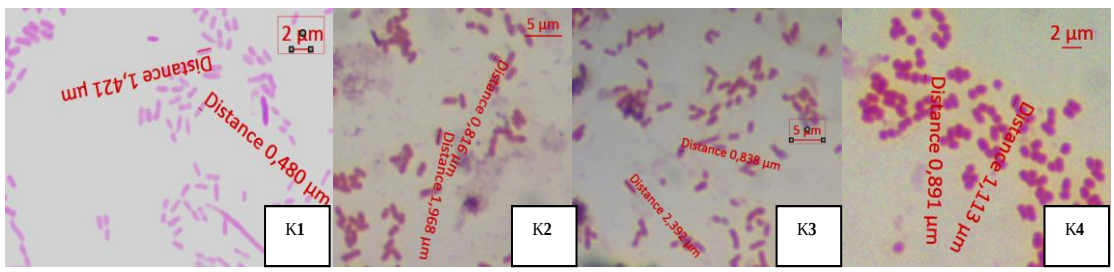


Fig. 2. Cell shape of bacterial strains isolated from potatoes' rhizosphere in Karo District.
K1: *Serratia marcescens*, K2: *Serratia* sp., K3: *Vibrio* sp., K4: *Aeromonas* sp.

Biofertilizer assays (determination of phosphate solubility and indole acetic acid/tryphtophanase) was negative for all four strains. In contrast, biopesticide assay (production of the proteolytic enzymes gelatinase, protease, and chitinase) results were positive for all strains, with the exception of *Aeromonas* sp., which was unable to synthesize gelatinase enzymes (Table 2). Biopesticide assays help determine the ability of a microorganisms to synthesize these proteolytic enzymes, which have an antagonistic role against other microorganisms. However, in this study *Aeromonas* sp. did not hydrolyse gelatine. *Aeromonas* species can either react positively or negatively during gelatine hydrolysis (Izuchukwu 2017, Torenzo et al. 1986)

Table 2. Biofertilizer and biopesticide assay results of selected antagonistic bacteria

Bacterial Strains	Biofertilizer assays		Biopesticide assays		
	Phosphate solubility	Indole acetic acid	Gelatinase	Protease	Chitinase
Serratia marcescens	-	-	+	+	+
Serratia sp.	-	-	+	+	+
Vibrio sp.	-	-	+	+	+
Aeromonas sp.	-	-	-	+	+

Nematode mortality did not differ significantly among the four bacterial strains (Table 3). More than 98% from the nematodes *Meloidogyne* spp. died after 4 hours of inoculation. The bacterial

mechanism of infection caused the lysis of nematode *Meloidogyne* spp., particularly on the esophageal region (*S. marcescens*) and body (*Serratia* sp.). In contrast, *Vibrio* sp. and *Aeromonas* sp. infected the nematodes until death, but they did not cause lysis and the nematodes' bodies were still intact (Table 4). Compared to the other strains, *Aeromonas* sp. caused the highest nematode mortality.

Table 3. Effect of antagonistic bacteria isolated from potatoes' rhizosphere on root knot nematode mortality.

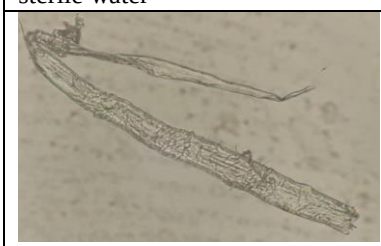
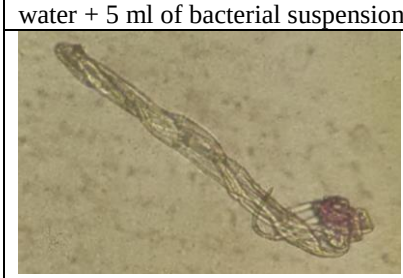
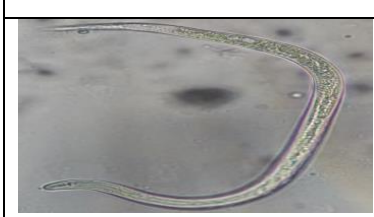
Treatments	**Percent mortality (%) of *Meloidogyne* spp Hours After Inoculation**			
	1	**2**	**3**	**4**
Serratia marcescens	4.80 [b]	16.80 [ab]	70.73 [c]	98.20 [a]
Serratia sp.	5.73 [ab]	16.87 [ab]	75.27 [b]	98.80 [a]
Vibrio sp.	5.86 [ab]	18.33 [a]	80.47 [a]	99.40 [a]
Aeromonas sp.	6.73 [a]	19.80 [a]	84.33 [a]	99.87 [a]
Control	0 [c]	0 [c]	0 [a]	0 [b]

Means followed by the same letter (superscript) for each number of mortality are not significantly different at P =0.05 according to Duncan's Multiple Range test.

The activity of each bacterial strains was evaluated based on the number of dead nematodes after 4 hours in the bacteria. Strains *S. marcescens*, *Serratia sp.* , *Vibrio* sp., and *Aeromonas* sp. demonstrated substantial biocontrol activity against the nematode *Meloidogyne* spp *in vitro*. The high percentage of nematode mortality proved their biocontrol activity affected the nematodes. The presence of all bacterial strains resulted in a nematodes' mortality of up to 98% after four hours of inoculation, with the highest mortality at 99.87% for *Aeromonas* sp. The highest nematode mortality was exhibited in *Aeromonas* sp., followed by *Vibrio* sp., *Serratia* sp., and then *S. marcescens*. For the control, which consisted only of sterile water, none of the nematodes died.

All bacteria isolated from the rhizosphere surrounding potatoes synthesized the lytic enzymes proteases and chitinases, while only *S. marcescens*, *Serratia* sp., and *Aeromonas* sp. strains synthesized gelatinase. This could have been due to the different concentrations of lytic enzymes acting as biocontrol agents against *Meloidogyne* spp. In general, pathogens are antagonized by the presence and activity of other organisms they encounter (Pal and Gardener 2006). Major secondary metabolites are derived from bacteria (Miao and Davies 2010). Different bacteria secrete and excrete metabolic substances that could affect a pathogen's growth and/or activities, including the synthesis of lytic enzymes, which can hydrolize many different polymeric compounds. Lytic enzymes are important in degrading various chemical constituents of nematodes during different developmental stages (Castaneda-Alvarez and Aballay 2016). Enzymatic activities are important in biocontrol in living organisms (Burns and Dicks 2002). Most bacteria excrete lytic enzymes, including glucanases, proteases, gelatinases, cellulases, and chitinases (Maheshwari 2013). The most potent bacterial isolates; i.e. *Pseudomonas fluorescens* and *Bacillus thuringiensis* exhibited maximum hydrolysis zone values for gelatinase, protease, and chitinase against *Meloidogyne incognita in vitro*, in accordance with the higest values of nematicidal activities from similar isolates (Zaghloul et al. 2015). Wang et al. (2009) showed that an extracellular alkaline protease (Hasp) had a potential useful factor in killing second stage juveniles of *Heterodera glycine* and also degraded nematode cuticle proteins. Microorganisms that can colonize and lyse plant pathogens may be categorized as biocontrol agents (Pal and Gardener 2006).

Table 4. Mechanism of antagonistic bacterial infection inside the body of *Meloidogyne* spp.

Microscopic nematode	Note
Control treatment: 100 nematodes (J2)/ 10 ml of sterile water	Nematodes were alive and moved actively.
 K1 treatment :100 nematodes (J2)/ 100 ml of sterile water + 5 ml of bacterial suspension ($1x10^6$ cfu)	Nematodes' stylet were lysed and broken near the esophageal (pharinx) region.
 K2 treatment:100 nematodes (J2)/ 100 ml of sterile water + 5 ml of bacterial suspension ($1x10^6$ cfu)	Nematodes' body region were lysed.
K3 treatment :100 nematodes (J2)/ 100 ml of sterile water + 5 ml of bacterial suspension ($1x10^6$ cfu)	Nematode was dead with an intact body.
 K4 treatment :100 nematodes (J2)/ 100 ml of sterile water +5 ml of bacterial suspension ($1x10^6$ cfu)	Nematode was dead with an intact body.

Although most rhizobacteria kill plant parasitic nematodes by means of direct antagonism, i.e. production of metabolic by-products (enzymes and toxins), rhizobacteria can also use other modes of action (Siddiqui and Mahmood 1999). They could suppress a nematode population by regulating nematode behavior (Sikora and Hoffmann-Hergarten 1993), interfering with nematode-plant recognition (Oostendorp and Sikora 1990), competing with nutrients (Oostendorp and Sikora 1990), promoting plant growth (El-Nagdi and Youssef 2004), and inducing systemic resistance (Hasky-Gunther et al. 1998, Seenivasan 2011).

S. marcescens was reported as a great potential biocontrol agent against root-knot nematode in field studies (Mohamed et al. 2009, Kamel et al. 2010, Abd-Elbagwed and Kabeil 2010). The *in vitro* experiment from their study showed that *S.marcescens* hydrolysed the enzymes gelatinase, protease, and chitinase, which have important roles as biocontrol agents against nematodes. Results revealed that the strains *S. marcescens* and *Serratia* sp. were not able to act as biofertilizers because of the negative results from the phosphate solubility and indole acetatic acid (IAA) tests. Thus, these strains might not have a direct role as plant growth promoting rhizobacteria once applied in a field trial. Maxton et al. (2017) also found that *S. marcescens* did not produce IAA *in vitro*. In contrast, the study of Kamel et al. (2010) reported the ability of *S. marcescens* to act as both a biocontrol agent and biofertilizer, as it exhibited a good response on the growth of faba plants.

Arshad and Frankenberger (2002) stated that the IAA production of bacterial isolates may vary, wherein the availability of precursors and the plant uptake of rhizospheric IAA regulates intrinsic IAA production of rhizospheric microorganisms. In the field, bacteria can stimulate chitinolytic enzyme activity, such as that of chitinases, to act as both a biocontrol agent and biofertilizer. Abiotic stress, such as the presence of heavy metals in soils, drought, nutrient deficiency, salinity, and temperature could stimulate the bacterial mechanism for improving crop yield (García-Fraile et al. 2015). Healthy crops may produce good quality of crops with higher yield and indirectly exhibit better resistance against plant pathogens, including nematodes.

On the contrary, no reports were found on the biocontrol activity of *Aeromonas* sp. and *Vibrio* spp. against nematodes. The chitinolytic bacteria (*Aeromonas caviae*) isolated from the rhizosphere of bean plants was able to suppress *Rhizoctonia solani* and *Fusarium oxysporum* f.sp. *vasinfectum* in cotton, and also suppressed *Sclerotium rolfsii* in beans (Inbar and Chet 1991). *Vibrio* sp. is commonly found in aquatic environments and are occasionally found in small amounts in wood substrates where earthworms exist (Magdoff and Weil 2004). Findings from this study provide new prospects for *Aeromonas* sp. and *Vibrio* sp. as biocontrol agents of root-knot nematodes. No previous studies have found that these two bacteria have a potential role to act as biocontrol agents against plant parasitic nematodes.

CONCLUSIONS

The study sought to isolate and screen bacteria from potatoes' rhizophere against root-knot nematode (*Meloidogyne* spp.). Four selected bacterial strains isolated from Karo district, North Sumatra, Indonesia, included the following: *Aeromonas* sp., *Vibrio* sp., *S. marcescens*, and *Serratia* sp. Root-knot nematodes reached up to more than 98% *in vitro* for the four bacterial strains. This suggests that these four bacteria can act as potential biological control agents managing root-knot nematodes in potatoes. However, further validation studies involving the bacterial application of these strains on potatoes infested with root-knot nematodes under both pot and field conditions need to be conducted.

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