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Screening of quorum quenching activity of rhizobacteria against *Pectobacterium carotovorum* subsp. *carotovorum*

SAFIRA RIZKA LESTARI[1,♥], FERY ABDUL CHOLIQ[2], ANTOK WAHYU SEKTIONO[2], MOCHAMMAD SYAMSUL HADI[2], HAIDAR FARI ADITYA[1], NONI RAHMADHINI[1], RAMADHANI MAHENDRA KUSUMA[1], YOGO SETIAWAN[2]

[1]Faculty of Agriculture, Universitas Pembangunan Nasional Veteran Jawa Timur. Jl. Rungkut Madya No. 1, Surabaya 60294, East Java, Indonesia.
[♥]email: safira.rizka.agro@upnjatim.ac.id
[2]Department Plant Pests and Diseases, Faculty of Agriculture, Universitas Brawijaya. Jl. Veteran, Malang 65145, East Java, Indonesia

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Abstract. *Lestari SR, Choliq FA, Sektiono AW, Hadi MS, Aditya HF, Rahmadhini N, Kusuma RM, Setiawam Y. 2022. Screening of quorum quenching activity of rhizobacteria against* Pectobacterium carotovorum *subsp.* carotovorum. *Biodiversitas 23: 4336-4342.* Quorum sensing (QS) is a mechanism for determining the virulence of the phytopathogenic bacterium *Pectobacterium carotovorum*. *P. carotovorum* carries out the QS mechanism with the chemical compound acyl-homoserine lactone (AHL) to signal and carry out its pathogenicity and virulence. The pathogenicity of *P. carotovorum* can be prevented through quorum quencher (QQ) activity derived from bacteria that degrade AHL compounds by producing AHL-lactonase. In the present investigation, AHL-lactonase-producing bacteria were isolated from the rhizosphere of carrot cultivation in Bumiaji Sub-District, City of Batu, East Java, Indonesia. The isolated bacteria were screened for quorum quenching activity. Out of thirty-seven isolates, 7 rhizospheric bacteria showed QQ activity against the *Chromobacterium violaceum* bioindicator. The hypersensitivity test on tobacco plants showed that, five strains SRL07, SRL09, SRL15, SRL21, and SRL35 did not cause a hypersensitivity response. The five isolates were able to suppress the virulence of *P. carotovorum* in carrot tubers. The 16S rRNA gene sequences of the five isolates had the highest similarity to *Bacillus thuringiensis*, *B. subtilis*, and *B. cereus*. The five isolates were detected to have the gene encoding AHL-lactonase (*aiiA*). The present study provides new information about the QQ activity of rhizosphere and its potential as a quorum quencher against *P. carotovorum*.

Keywords: AHL-lactonase, biocontrol, *Pectobacterium carotovorum*, quorum quenching

INTRODUCTION

Quorum sensing (QS) is a bacterial mechanism for sharing information among cells that depends on the density of bacterial colonies (Rutherford and Bassler 2012). The function of QS is to convey signals in the form of chemical compounds to bacterial colonies (Zhao et al. 2020; Pangastuti et al. 2021). Chemical compounds that act as signals in QS, in general, include (1) acyl-homoserine lactones (AHLs), (2) auto-inducing peptides (AIPs), and (3) auto-inducer 2 (AI-2) (Huang et al. 2016). Gram-negative plant pathogenic bacteria carry out the QS mechanism with the chemical compound acyl-homoserine lactone (AHL) as a signal to carry out their pathogenicity and virulence (LaSarre and Federle 2013).

Pectobacterium carotovorum subsp. *carotovorum* is a Gram-negative and common soil-borne bacterium that causes soft rot disease of various plant hosts, such as vegetables and fruits (Abd-El-Khair et al. 2021). *P. carotovorum* is the cause of soft rot disease in carrots, potatoes, lettuce, onions, and cabbage (Lee et al. 2016; Fan et al. 2017). *Pectobacterium carotovorum* employs 3-oxo-hexanoyl homoserine lactone (3OC6HSL) to regulate the expression of genes encoding virulence factors such as pectate lyases, proteases, and cellulase enzymes (Garge and Nerurkar 2016). In Indonesia, *P. carotovorum* also attacks carrot plants. One of the obstacles in carrot cultivation is the soft rot disease caused by the phytopathogenic bacteria *P. carotovorum* (Marquez-villavicencio et al. 2011; Máisuría and Nerurkar 2013). Soft rot disease causes potatoes to be watery, mushy, and blackish, making them unsuitable for sale. Currently, management efforts to control soft rot disease that have been carried out involve applying synthetic bactericides, performing minimum tillage, eradicating, and irradiating with LED lights.

Another strategy that can be implemented is to control the pathogenicity of *P. carotovorum* by utilizing the quorum quenching (QQ) mechanism (Fan et al. 2020; Zhang et al. 2020). The QQ mechanism is a QS prevention or inhibition mechanism. The QQ mechanism can be carried out in three ways: degradation and inactivation of QS signal molecules, inhibition of the biosynthesis of QS signal molecules, and inhibition of detection of QS signal molecules (Natrah et al. 2011). QQ inactivates AHL in expressing genes encoding virulence factors in phytopathogens (Baltenneck et al. 2021). Romero et al. (2008) and Utari et al. (2017) reported that specific plant cells and certain bacterial cells could inhibit QS by producing compounds with structures similar to AHL but act as the inhibitors and produce AHL-degrading enzymes, namely AHL-lactose. The QQ mechanism is considered safer for controlling phytopathogens without usage of bactericides because it does not cause resistance to phytopathogens (Rehman and Leiknes 2018).

The rhizosphere is a habitat of various microorganisms, including archaea, bacteria, and fungi that possess the QQ mechanism (Ghosh and Mandal 2022). Rhizobacteria that are known to have QQ activity include *Acinetobacter* sp., *Burkholderia* sp., *Klebsiella* sp., *Bacillus aquimaris, B. marisflavi, B. altitudinis*, and *B. axarquiensis* (Chan et al. 2011; Fitriyah et al. 2015). Bacteria that have QQ activity were reported to control the pathogenicity of *Ralstonia solanacearum*, *Dickeya dadantii*, and *Pseudomonas aeruginosa, in vitro* (Khoiri and Damayanti 2017; Jayanna and Umesha 2017; Rehman and Leiknes 2018). A study on QQ activity in rhizobacteria was carried out and indicated that rhizobacteria produce AHL-lactose (Prazdnova et al. 2022). Information on the potential of rhizobacteria as a quorum quencher in Indonesia still needs to be further discovered and developed. The present investigation deals with isolation of rhizospheric bacteria and characterization of rhizobacteria for quorum quenching activity against *Pectobacterium carotovorum* subsp. *Carotovorum.*

MATERIALS AND METHODS

Soil sampling and isolation of rhizobacteria

Soil samples were collected from carrot fields that indicated the spread of *P. carotovorum* soft rot disease in Bumiaji Sub-District, City of Batu, East Java, Indonesia. Soil samples were taken from five sampling points by the diagonal method to a depth of 10 cm from the top soil. The distance between the sampling points was approximately 50 m, measured from the center point. Isolation of rhizobacteria was conducted at the Plant Diseases Laboratory of National Development University "Veteran" East Java. Isolation of rhizobacteria was carried out using serial dilution with the spread plate method on nutrient agar (NA) media. One gram of soil sample was suspended in 9 mL of 0.85% NaCl solution. The suspension was diluted up to 8-10 dilutions. The suspension (100µL) from the last three dilutions was spread onto NA plates and then incubated at room temperature (28-30°C) for 48 h. Bacterial colonies that grew and showed different morphological characteristics were purified by the quadrant streak plate method on the NA plates.

Chromobacterium violaceum detection of QQ activity

The purified bacterial isolates were grown in Luria-Bertani (LB) broth and shaken for ±18 h until the OD_{600} value reached 0.8. The culture was centrifuged at 16,000 g for 10 min. The supernatant was taken and filtered with a 0.22 µm syringe filter. A hundred milliliters of the supernatant filtrate were dripped onto a sterile paper disc before being placed on the surface of semi-solid LB agar plate that had been inoculated with *C. violaceum* (OD_{600} = 0.8) 1% (v/v) culture and incubated at room temperature (28-30°C) for 24 h. LB plate dripped on a sterile paper disc was utilized as a negative control. AHL degradation activity was indicated by the presence of a non-purple zone around the paper disc (Fitriyah 2015).

Pathogenicity test of AHL-degrading rhizobacteria on tobacco plants

Selected bacterial isolates and *P. carotovorum* were grown on NB media. The culture was incubated until the OD_{600} value reached 0.8. Infection of bacterial suspension (0.5 mL) on tobacco plants was carried out with a syringe that was not fitted with a needle (infiltration). The leaves were infiltrated with 0.5 mL of sterile NB for negative control culture and for a positive control, the leaves were infiltrated with 0.5 mL of *P. carotovorum* suspension. Each treatment was repeated three times. Necrotic symptoms that appeared on the leaf tissue were observed on the second day after treatment.

In vitro pathogenicity inhibition test for *Pectobacterium carotovorum*

The *in vitro* pathogenicity inhibition test was carried out by soaking potatoes in a 70% alcohol solution for 2 min and sterile distilled water for 2 min, before being exposed to UV light for 15 min. The potatoes were pierced with a sterile toothpick to a depth of 5 mm and coated with selected bacterial isolates. The potatoes were coated by rolling the tubers in a dish containing a liquid culture of a bacterial isolate (OD_{600} = 0.8). Next, the potatoes were dried in a laminar flow cabinet. Hundred microliters culture of *P. carotovorum* (OD_{600} = 0.8) were dripped into the puncture hole. Potatoes for positive control were stabbed and had *P. carotovorum* culture dripped into them without being smeared with the selected isolate culture, and potatoes for negative control were pierced and had sterile NB media dripped into them without being smeared with the selected isolate culture first. Each treatment was repeated three times. The tubers were then incubated in a plastic box for 24 h at room temperature (28-30°C). After incubation, the potatoes were cut into two halves precisely in the center of the puncture hole, and the width and depth of the rotting tissue were measured. The severity of soft rot was measured based on the area of soft rot.

Antibiosis test of AHL-degrading rhizobacterial isolates

Selected bacterial isolate cultures (OD_{600}=0.8) were centrifuged at 16,000 g for 10 min. The supernatant was sterilized with a 0.22 µm pore diameter membrane filter. Fifteen microliters of the filtrate were dripped on a sterile paper disc and then placed on the surface of NA media that had been spread with *P. carotovorum* culture (OD_{600} = 0.8). Sterile paper discs containing 15 µL of sterile NB plates were utilized as negative controls, while paper discs containing 400 ppm ampicillin were utilized as positive controls. Each treatment was repeated twice. Incubation was carried out at room temperature for 24 h. Antibiotic activity was indicated by the formation of a clear zone around the paper disc.

Morphological characterization and identification of AHL-degrading bacteria

Selected QQ bacterial isolates were characterized based on the colony and cell morphology. Bacterial genomic DNA isolation was carried out following the standard procedure of the Presto™ Mini gDNA Bacteria

Kit (Geneaid, USA). The 16S rRNA gene amplification was performed with usage of primers 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'-GGGCGGWGTGTACAAGGC-3') (Marchesi et al. 1998). The final concentration of the PCR reaction consisted of 1 µL of GoTaq® Green Ready Mix (Promega, USA), 0.5M of each primer, 10 µg of genomic DNA, and nuclease-free water up to a volume of 50 µL. The PCR steps were carried out, involving pre-denaturation at 94°C for 5 min, denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, elongation at 72°C for 30 sec, and final elongation at 72°C for 7 min. The amplification process was carried out for 30 cycles. The PCR products were migrated on 1% agarose gel at 80 V for 45 min. DNA visualization was performed with a UV transilluminator with EtBr staining. Determination of the nucleotide sequence was conducted by sending the amplified DNA to a sequencing service provider. The results of the sequencing were then analyzed with the SeqTrace software. The obtained sequences were aligned with the data on GenBank® by the Basic Local Alignment Search Tool nucleotide (BLASTn) program from the National Center for Biotechnology Information (NCBI) website at http://www.ncbi.nlm.nih.gov. The phylogenetic tree construction was carried out by the neighbor-joining (NJ) method with MEGA 6.0 software. The topology of the phylogenetic tree construction was evaluated through 1,000-iteration bootstrap analysis.

Detection of AHL-lactonase (aiiA) coding gene

The amplification of the gene encoding AHL-lactonase (*aiiA*) was carried out with usage of *aiiA* gene-specific primers that comprise aiiAF (5'-TCGGATCCATGACAGTAAAGAAGCTTATTTCG-3' and aiiAR (5'-GTCGAATTCCTCAACAAGATACTCCT AATGATGT-3') (Marchesi et al. 1998). The final concentration of the PCR reaction consisted of 1 µL of GoTaq® Green Ready Mix (Promega, USA), 0.5M of each primer, 10 ng of genomic DNA, and nuclease-free water up to a volume of 50 L. The PCR steps involved pre-denaturation at 94°C for 5 min, denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, elongation at 72°C for 30 sec, and final elongation at 72°C for 7 min. The amplification process was carried out for 30 cycles. The PCR product was migrated to 1% agarose gel at 80 V for 45 min. DNA visualization was carried out with a UV transilluminator by EtBr staining. The determination of the nucleotide sequence was carried out by sending the amplified DNA to a sequencing service provider. The results of the sequencing were then analyzed with usage of the SeqTrace software. The obtained sequences were aligned with the data on GenBank® by the BLAST nucleotide with the six-frame translation protein (BLASTx) program from the NCBI web site.

Data analysis

The data of soft rot diameter and relative inhibition in this study were analyzed with ANOVA, and if the results were significantly different among the treatments, a post-hoc test was conducted with the Duncan Multiple Range Test (DMRT) with an error level of 5%. Data were analyzed with usage of the SPSS program, version 22.0.

RESULTS AND DISCUSSION

Bioindicator-based detection of QQ activity

In the present investigation, 37 rhizobacteria were isolated from carrot fields, where the spread of *P. carotovorum* soft rot disease was indicated. Seven isolated rhizobacteria were able to show their potential to inactivate signal compounds produced by *C. violaceum* as bioindicators. Isolates of rhizobacteria that have the potential to inactivate signal compounds produced by *C. violaceum* as bioindicators were able to form a clear zone around their colonies. Degradation activity of seven AHL-degrading rhizobacteria (supernatant) with *C. violaceum* bioindicators was reported (Figure 1, Table 1).

Hypersensitivity reaction of AHL-degrading rhizobacteria isolates on tobacco plants

Two of the seven isolates of AHL-degrading rhizobacteria showed a hypersensitive response. The hypersensitivity response is characterized by the presence of yellow spots on tobacco plants (Figure 2).

QQ activity of AHL-degrading rhizobacteria isolates in vitro

The results showed that strain SRL35 had the smallest diameter of soft rot, while the largest was for the control. The isolate of rhizobacteria that was most able to inhibit the virulence of *P. carotovorum* was the strain SRL35, which had a relative inhibition of 65.25%. The lowest relative inhibition level was for strain SRL09 (41.96%) (Table 2).

Table 1. Diameters of clear zones for isolates of AHL-degrading rhizobacteria

Bacterial strain	Clear zone diameter (mm)
SRL07	14.78
SRL09	16.15
SRL14	17.03
SRL15	18.10
SRL21	16.00
SRL33	14.60
SRL35	18.30

Table 2. Development of soft rot symptoms caused by *Pectobacterium carotovorum* in carrot slices

Treatment	Diameter soft rot (mm)	Relative inhibition of soft rot (%)
Pectobacterium carotovorum (Control)	5.48^{d}	00.00^{a}
Pectobacterium carotovorum + SRL07	2.45^{b}	55.22^{b}
Pectobacterium carotovorum + SRL09	3.18^{c}	41.96^{a}
Pectobacterium carotovorum + SRL15	2.73^{b}	50.18^{a}
Pectobacterium carotovorum + SRL21	2.38^{b}	56.53^{b}
Pectobacterium carotovorum + SRL35	1.90^{a}	65.25^{c}

Note: According to Duncan's test, mean values followed by same letters in each row are not significantly different at P<0.05.

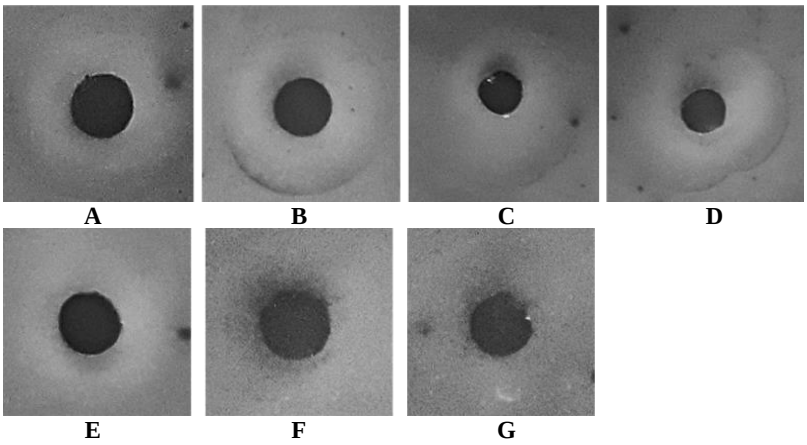

Figure 1. Degradation activity of seven AHL isolates of rhizobacteria (supernatant) with *Chromobacterium violaceum* bioindicators: a) Isolate SRL07; b) Isolate SRL09; c) Isolate SRL14; d) Isolate SRL15; e) Isolate SRL21; f) Isolate SRL33; g) Isolate SRL35

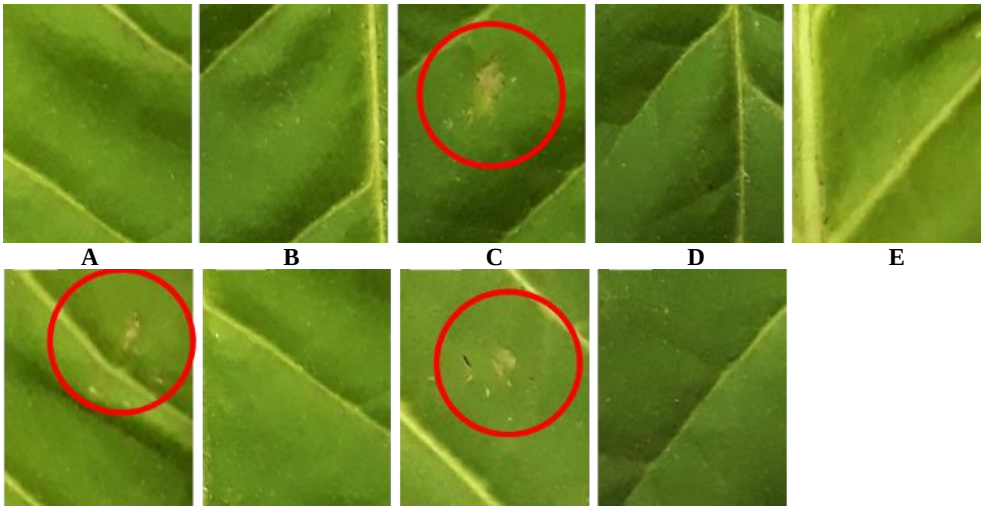

Figure 2. Hypersensitivity test of AHL-degrading rhizobacteria isolates on tobacco plants: A. SRL07 isolate; B. Isolate SRL09; C. Isolate SRL14; D. Isolate SRL15; E. Isolate SRL21; F. Isolate SRL33; G. Isolate SRL35; H. *Pectobacterium carotovorum* (positive control); I. Sterile NB (negative control). Red circles indicate necrotic tissue caused by a hypersensitive response

Antibiosis of AHL-degrading rhizobacterial isolates

The five isolates of rhizobacteria showed no antibiotic activity against *P. carotovorum*. This is indicated by the absence of a clear zone around the paper disc containing isolates of AHL-degrading rhizobacteria against *P. carotovorum*, which had been spread on the surface of the agar culture media (Figure 3).

Morphological characteristics of AHL-degrading rhizobacterial isolates

The five selected rhizobacterial isolates had varied colony morphological characteristics when grown on NA plate. The results of Gram staining showed that the five selected isolates belonged to the group of Gram-positive bacteria and had rod-shaped cells. However, there were differences in the shape, color, and edges of each isolate of AHL-degrading rhizobacteria (Table 3).

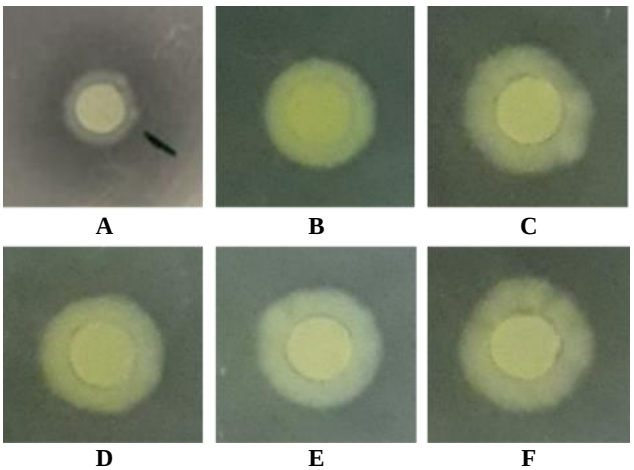

Figure 3. Antibiotic activity test of AHL-degrading rhizosphere isolates against *Pectobacterium carotovorum*: A. Ampicillin 400 ppm; B. Isolate SRL07; C. Isolate SRL09; D. Isolate SRL15; E. Isolate SRL21; f) Isolate SRL35

Identification of AHL-degrading rhizobacteria based on the 16S rRNA gene

Analysis of the 16S rRNA gene showed that there were great similarities of AHL-degrading rhizosphere isolates SRL35 and SRL07 with *B. subtilis*, isolate SRL09 with *B. thuringiensis*, isolate SRL21 with *Bacillus* sp., and isolate SRL15 with *B. cereus* (Figure 4 and Table 4).

Detection of AHL-lactonase (*aiiA*) coding gene

The detection results showed that the five isolates were positive for the aiiA gene. The results of gene analysis with the BLASTx program showed that the protein encoded by the aiiA gene of the SRL35 isolate was 99% similar to AHL-Lactonase in B. subtilis, the aiiA gene of the SRL21 isolate was 99% similar to AHL-Lactonase in B. thuringiensis, the aiiA gene of the SRL15 isolate was 98% similar to AHL-Lactonase in B. cereus, the aiiA gene of the SRL09 isolate was 99% similar to AHL-Lactonase in B. thuringiensis, and the aiiA gene of the SRL21 isolate was 99% similar to AHL-Lactonase in B. subtilis (Table 5).

Table 3. Morphological characteristics of five selected AHL-degrading rhizobacterial isolates

Bacterial strain	Colony morphology					Cell shape	Gram
	Shape	Color	Surface	Edge	Width (mm)		
SRL07	Round	Crème	Convex	Entire	1-2	Rod	+
SRL09	Irregular	White	Convex	Undulate	2-7	Rod	+
SRL15	Round	Yellowish	Convex	Entire	1	Rod	+
SRL21	Irregular	Yellowish	Convex	Undulate	2-5	Rod	+
SRL35	Round	White	Convex	Entire	1-2	Rod	+

Table 4. Identification of selected bacterial strains

Bacterial strain	Homology (%)	Species database on GenBank	Accession no.
SRL07	97	*Bacillus subtilis*, BS01 (HM631975)	K778902
SRL09	99	*Bacillus thuringiensis*, NCIMB9134 (X55062)	K778903
SRL15	98	*Bacillus cereus*, MBG23 (JF280125)	K778904
SRL21	99	*Bacillus* sp. strain MBG01 (JF280118)	K778905
SRL35	99	*Bacillus subtilis*,BS02 (HM631974)	K778906

Table 5. Identity of the *aiiA* gene of QQ bacteria

Isolates	Homology (%)	Species Identity of *aiiA* Gene
SRL07	97	*Bacillus subtilis* BS1 (DQ000640)
SRL09	99	*Bacillus thuringiensis* HQ21 (FJ172765)
SRL15	98	*Bacillus cereus* MBG31 (JF501490)
SRL21	99	*Bacillus thuringiensis* KACC12061 (GU339168)
SRL35	99	*Bacillus subtilis* BS1 (DQ000640)

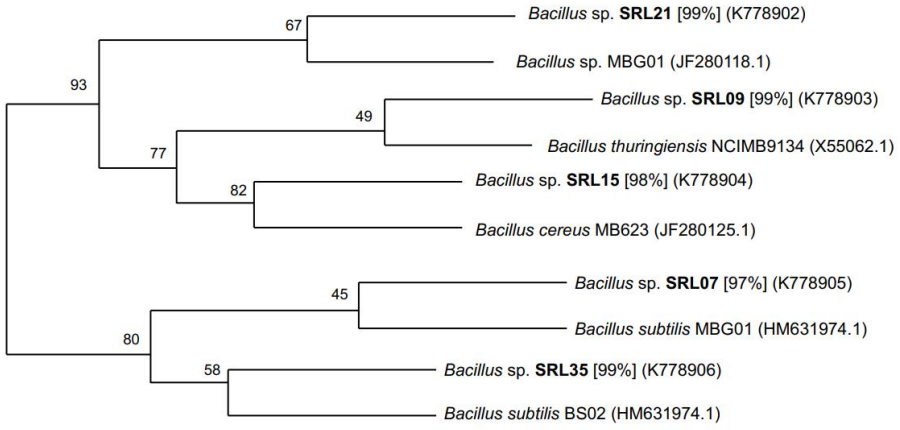

Figure 4. Construction of a phylogenetic tree with the statistical method of Neighbor-Joining Method and Jones-Taylor-Thorthon (JTT) substitution model with 1000x repetition

Discussion

Rhizobacteria that were collected for this study were able to show their potential to inactivate signal compounds produced by *C. violaceum* as bioindicators. This is shown by the fact that during the QS process, *C. violaceum* produces violaceum pigment. This is in accordance with Kothari et al. (2017) in that if the QS process fails, then *C. violaceum* does not produce the pigment. The failure of the QS process was caused by the AHL-degrading enzyme that was excreted by the rhizobacterial isolates. The enzyme is able to degrade the compound N-hexanoyl homoserine lactone (HHL), which is a signal compound for the QS process in *C. violaceum* (McClean et al. 1997).

In the present study, the highest QQ activity was shown by *Bacillus subtilis* SRL35, while the lowest activity was shown by *Bacillus thuringiensis* SRL09. The difference in the results of soft rot inhibition on carrot tubers could be caused by several internal factors such as enzyme production, bacterial colony growth, enzyme effectiveness, and environmental factors such as temperature and acidity. The best activity of an AHL-degrading enzyme is the AHL-lactonase from *Bacillus* sp. strain B546, *Bacillus* sp. strain A196, and *Bacillus weihenstephanensis* P65 at pH 6-9 (Chen et al. 2010; Cao et al. 2012; Sakr et al. 2013). Similarly, pure AHL-lactonase activity was at pH 6-9 (Dong et al. 2018). However, Eka et al. (2016) in another study reported that AHL-lactonase from *B. thuringiensis* SGT3g was active in a more acidic pH range, being 5-8. This indicates that the activity of QQ is influenced by the suitability between internal factors, such as the characteristic of the produced enzyme, and environmental factors, such as the acidity around the place where the QQ bacteria grow.

AHL-degrading rhizobacteria were able to inhibit the QS process enzymatically. Several enzymes that are known to degrade and inactivate AHL molecules belong to two classes: Class I enzymes, which break down AHL molecules, consisting of AHL-lactonase, AHL-acylase, and paraoxonase; and Class II enzymes, which reduce carbonyl to hydroxyl, consisting of oxidoreductases (Chen et al. 2013). AHL-degrading rhizosphere isolates *B. subtilis* SRL35 and *B. subtilis* SRL07 had the highest similarity with *B. subtilis*, while *B. thuringiensis* SRL09 showed similarity with *B. thuringiensis*, *Bacillus* sp. Strain SRL21 showed similarity with *Bacillus* sp., and *Bacillus cereus* SRL15 showed similarity with *B. cereus.* Chen et al. (2013); Khoiri et al. (2016); and Rehman and Leiknes (2018) reported that *Bacillus* is a genus of AHL-degrading bacteria commonly found in the rhizosphere. *Bacillus* can produce lactonase, cleave the lactone ring from the acyl moiety of AHL, and render AHL inactive in signal transduction (Chen et al. 2013). The QQ process can be carried out through the enzymatic degradation of AHL by the AHL-lactonase enzyme. AHL-lactonase can hydrolyze ester bonds in the AHL lactone ring (Tang et al. 2015). The *aiiA* gene is one of the genes encoding AHL-lactonase. The detection results showed that the five isolates were positive for the *aiiA* gene. This is in accordance with the research reported by Chen et al. (2013); Khoiri et al. (2016); and Rehman and Leiknes (2018), in that the gene encoding the AHL-lactonase enzyme in the *Bacillus* genus is *aiiA*.

In conclusion, five of the 37 isolates of AHL-degrading bacteria from the rhizosphere of Bumiaji Sub-District, Batu City, East Java had the potential as quorum quencher bacteria and plant disease biocontrol agents because they do not cause hypersensitivity responses on tobacco. The QQ activities of the five isolates were proven to be able to inhibit the pathogenicity of *P. carotovorum.* Based on the analysis of the 16S rRNA gene sequence, the five isolates were identified as *Bacillus* genus i.e., *B. subtilis*, *B. thuringiensis*, and *B. cereus.* The present study provides new information about the QQ activity of rhizosphere and its potential as a quorum quencher against *P. carotovorum.*

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