



AHL-lactonase characteristics of *Bacillus thuringiensis* SGT3g and its effectiveness in inhibiting pathogenicity of *Dickeya dadantii*

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ABSTRACT

Aims: *Dickeya dadantii* is a pathogenic bacterium causing bacterial soft rot disease in plants. The bacterium uses a homoserine lactone signal in its quorum sensing process to express the virulence factor genes. Anti-quorum sensing is a new approach to control plant pathogenic bacteria. The aims of this study are to characterize AHL-lactonase enzyme produced by *Bacillus thuringiensis* SGT3g and to determine its effectiveness in inhibiting virulence of *D. dadantii*.

Methodology and results: Activity of AHL-lactonase was determined using *Chromobacterium violaceum* as a bacterial biosensor. The crude extract enzymes of AHL-lactonase on both as extracellular and intracellular enzymes were analyzed their enzyme activity of protein precipitation and dialysis products. The optimum activity of AHL-lactonase was found at 30 °C and pH 5-8. *Bacillus thuringiensis* SGT3g was capable to reduce soft rot symptom disease caused by *D. dadantii* on *Phalaenopsis* orchid leaves after 24 h of incubation.

Conclusion, significance and impact study: *Bacillus thuringiensis* SGT3g was capable to degrade AHL signal of *C. violaceum* and *D. dadantii*. The activity AHL-lactonase of *B. thuringiensis* SGT3g had a wide range of pH and temperature. The lactonase could reduce soft rot symptom disease caused by *D. dadantii* without any growth inhibition of *D. dadantii* on orchid leaves. *Bacillus thuringiensis* SGT3g can be used as an alternative biopesticide to control phytopathogenic bacteria due to its capability to suppress bacterial pathogenic virulence.

Keywords: AHL-degradation, *Bacillus thuringiensis*, *Phalaenopsis*, quorum sensing, quorum quenching

INTRODUCTION

Communication mechanisms among bacterial cells are known as quorum sensing (QS). This mechanism involves an auto-inducer as a signal molecule. During the quorum sensing process, auto-inducers are secreted onto environment then accumulated, being recognized and re-entering the cells of the bacteria (Czajkowski and Jafrá, 2009; Sakr *et al.*, 2013). In certain concentration, auto-inducers will form a complex with transcription-activating regulatory proteins and eventually control genes expression of biofilm formation, virulence factors, bioluminescent, and antibiotics production. In general, QS is a gene expression regulation that depends on the number of bacterial population and auto-inducers accumulation (Dong *et al.*, 2007).

Plant pathogenic bacteria use the QS process to activate their virulence factors. Phytopathogenic bacteria such as *Dickeya dadantii* or *Erwinia chrysanthemi* use N-acyl homoserine lactone (AHL) signal to control gene expression of their virulence factors. The AHL signal has a significant role in virulence factor process of *D. dadantii*

to produce Plant Cell-Wall Degrading Enzyme (CWDE), i.e pectinase and cellulase (Barnard *et al.*, 2007; Muharram *et al.*, 2012). Activity of the pectinolytic and cellulolytic enzymes is to degrade plant cell wall (Dong *et al.*, 2000; Samson *et al.*, 2005; Barnard *et al.*, 2007). This bacterium causes soft rot diseases in orchids, cabbages, and carrots (McMillan *et al.*, 2007; Muharram *et al.*, 2012).

Process of QS can be controlled in various ways, including: inhibiting AHL biosynthesis, destroying QS signal molecules through the activities of AHL-lactonases and AHL-acylases, the inhibition of AHL efflux proteins, inhibition of transcriptional activators (LuxR homologs) and the use of QS analogs (Kalia *et al.*, 2015). Destroying QS signal through the activities of AHL-lactonases can inhibit accumulation of AHL signals surrounding the bacterial cells (Chen *et al.*, 2013; Ghani *et al.*, 2014; Kalia *et al.*, 2015). AHL-lactonase hydrolyzes the lactone ring of N-(pentanoyl) homoserine lactone (C5HSL), N-(decanoyl) homoserine (C10HSL), and a few synthetic compounds [N-tert-butylloxycarbonyl]-DL-homoserine lactone and N-(benzyloxycarbonyl)-L-homoserine lactone] with high efficiency (Kumar *et al.*, 2013). As a result, the enzyme

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can inhibit genes expression of virulence factor without any effect to the growth of the pathogenic bacteria. Thus it can avoid the selection pressure that can generate bacterial resistant mechanisms of the pathogenic bacteria (Dong *et al.*, 2004; Choudhary and Dannert, 2010).

AHL-lactonase was initially found in *Bacillus* sp. 240B1 by Dong *et al.* (2001) which is capable to reduce soft rot disease caused by *E. carotovora* in potato plants. Bacteria from various genera which are capable to degrade AHL are *Bacillus* sp., *B. thuringiensis*, *Arthrobacter* sp., and *Rhodococcus* sp. (Dong *et al.*, 2002; Park *et al.*, 2003; Wang *et al.*, 2004; Park *et al.*, 2006). The activity of AHL-lactonase is affected by several factors, such as temperature, pH, and divalent ions. AHL-lactonase of *B. weihenstephanensis* has the optimum activity at 50 °C and pH range of 6-9, it is also unaffected by divalent ions i.e. Ca²⁺, Mg²⁺, and Fe²⁺ (Sakr *et al.*, 2013).

Afiah (2011) isolated *B. thuringiensis* SGT3g producing AHL-lactonase. AHL-lactonase characterization of *B. thuringiensis* SGT3g is necessary in order to determine the enzyme activity and stability against pH and temperature. Therefore, in this study we describe the characteristics of AHL-lactonase of *B. thuringiensis* SGT3g AHL-lactonase, and its effectiveness in inhibiting the virulence of phytopathogenic bacterium *D. dadantii*.

MATERIALS AND METHODS

Crude enzyme extraction

Bacillus thuringiensis SGT3g isolated from agriculture land at Java Island, Indonesia (Afiah, 2011), was grown on Nutrient Agar medium. A total of 2 mL of *B. thuringiensis* SGT3g culture with OD of 0.6-0.8 was inoculated into 200 mL LB media. Incubation was done on a shaker incubator at 150 rpm and at room temperature for 9 h. The culture was centrifuged at 9800 $\times g$, to obtain supernatant as an extracellular crude enzyme; and the cell pellet as source of intracellular crude enzyme. To obtain intracellular crude enzyme, cell pellet was washed using Phosphate Saline Buffer (PBS) pH 7.4 and then centrifuged at 4 °C and 6000 rpm for 10 min. After pellet re-suspension using PBS, the cells was lysed using sonicator for 2 min in 5 times replications. The cell debris was separated by centrifugation at 12500 $\times g$ for 15 min and the supernatant was collected as intracellular crude enzyme (Dong *et al.*, 2002; Cao *et al.*, 2012).

Bioassay of AHL degrading activity

Activity of AHL-lactonase was determined using disc diffusion assay (Fitriyah *et al.*, 2014). A total of 80 μ L crude extract enzyme was dropped on a paper disc and placed on a plate of semi-solid Luria-Bertani Agar (LBA) media that had been inoculated with 1% culture of *C. violaceum* (10⁸ CFU/mL). The plate was then incubated at room temperature for 24 h. AHL degradation activity was determined by measuring the diameter of a non-purple zone surrounding the paper disc.

Enzyme purification and characterization

AHL-lactonase was precipitated using ammonium sulphate with 40-80% (b/v) saturation value (Scopes, 1994). After being precipitated overnight at 4 °C, the enzyme then centrifuged at 10000 $\times g$ for 15 min. The obtained pellet was then dissolved in 0.1 M citrate buffer pH 5 and the dialysis process was carried out using 15 mm (diameter) dialysis bag (Sigma D0405). The precipitated enzyme was dissolved in 1 mL citrate buffer 50 mM pH 5.0. The enzyme solution was then dialyzed using dialysis bag with 12.5 mM citrate buffer, and stirred using a magnetic stirrer at 4 °C. The dialysis process was carried out overnight and 12.5 mM citrate buffer was replaced with new buffer solution after 3 and 7 h of incubation.

Characterization of the precipitated enzyme was conducted based on pH and temperature treatments. The buffers pH used for characterization were 0.1 M citrate buffer for pH range of 4.0-6.0, 0.1 M phosphate buffer for pH range of 7.0-8.0 and 0.1 M glycine-NaOH buffer for pH range of 9.0-10.0. Meanwhile the optimum temperature was determined at temperature range of 20-90 °C with interval of 10 °C for every treatment (Sakr *et al.*, 2013).

Protein concentration measurement

Protein concentration was measured using the method of Bradford (1976). A total of 2.5 mL Bradford reagent was mixed with 50 μ L crude extract enzyme and the suspension was homogenized using vortex then incubated for 10 min. The absorbance value was measured using spectrophotometer at 595 nm wavelength.

In planta inhibition assay of *B. thuringiensis* SGT3g against *D. dadantii*

This assay was conducted using hybrid orchid *Phalaenopsis ekanagasaki* provided from Bogor Botanical Garden, Indonesia. The orchid leaves were smeared with 3 mL *B. thuringiensis* SGT3g culture of 10⁸-10⁹ CFU/mL using a sterile brush. After the surface of leaves dried, each leaf sample was covered using a plastic bag and they were incubated for 24 h. Then the leaves were stabbed using a sterile needle for 10 times. A total of 5 μ L *D. dadantii* (10⁸-10⁹ CFU/mL) was flooded onto each injection spot which was the Treatment I or the not co-culture method. Treatment II was a mix culture (co-culture) of *B. thuringiensis* SGT3g (10⁸-10⁹ CFU/mL) with *D. dadantii* (10⁸-10⁹ CFU/mL), a total of 3 mL mix culture was dripped onto each injection spot. The positive control of the assay is *D. dadantii* culture, while the negative control is *B. thuringiensis* SGT3g culture. After inoculation, the plants were covered using a plastic bag and incubated overnight. The occurrence of the disease symptoms (the number of spots with symptoms divided with total inoculation spots) and the development of

disease symptoms (the diameter of the symptoms) were observed.

Twenty hours after inoculation of the pathogen, the samples of orchid leaves, positive control leaves, and treatment leaves were cut, macerated, and serially diluted. The dilution results were spread on LA plate which was added with 0.5% crystal violet and 25 ppm ampicillin. *D. dadantii* colony was counted after 24 h incubation.

RESULTS

Activity of *B. thuringiensis* SGT3g AHL-lactonase crude enzyme

Activity of AHL-lactonase crude enzyme was detected in the supernatant and cell pellet extract (Table 1). This activity was indicated by the formation of non-purple zone around the paper disc. The results showed that the intracellular enzyme had the highest activity index of non-purple zone formation than that of the extracellular enzyme.

Table 1: Crude extracts enzyme activity of *B. thuringiensis* SGT3g AHL-lactonase indicated by non-purple zone formation of *C. violaceum* bacterial colony.

Enzyme Source	Non-purple Zone (mm)
Supernatant	7.25 ± SE 0.265
Pellet	10 ± SE 0

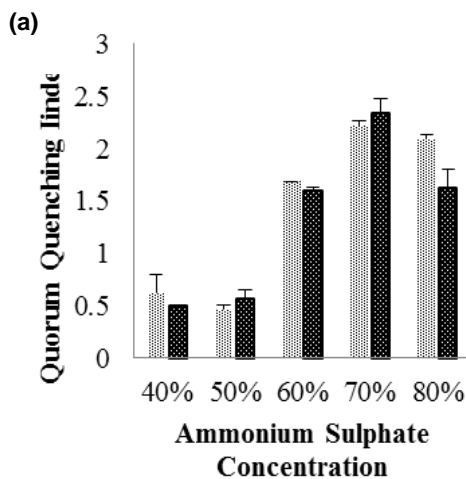
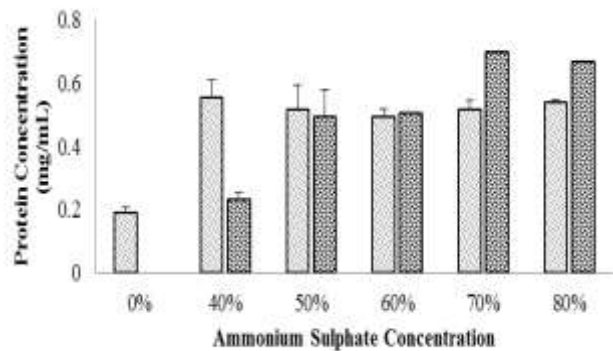


Figure 2: a, Quorum quenching index of *B. thuringiensis* SGT3g AHL-lactonase crude extract (stippled, supernatant; solid black, precipitated protein) and b, comparison of quorum quenching index between intracellular and extracellular enzymes (stippled, supernatant; solid black, precipitated protein).

The activity and stability of extracellular AHL-lactonase produced by *B. thuringiensis* SGT3g was characterized based on pH and temperature treatments. The results indicated that the enzyme had good stability in a wide range of pH and temperatures (Figure 3). However

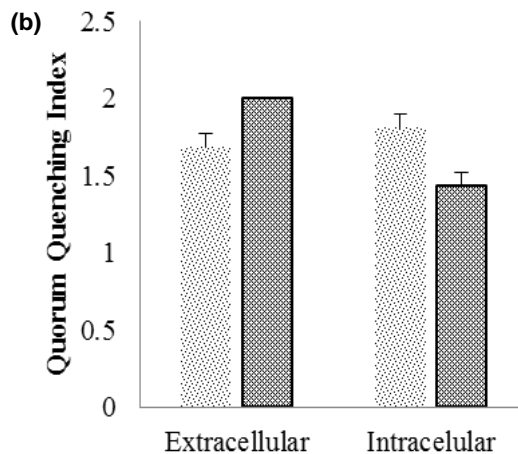
Characteristics of precipitated AHL-lactonase of *B. thuringiensis* SGT3g

The data of protein precipitation showed that the highest precipitated protein concentration was at 70% (w/v) ammonium sulphate (Figure 1). The data of quorum quenching index indicated that salting-out of AHL-lactonase was occurred at 70% (w/v) ammonium sulphate (Figure 2a). The quorum quenching index of the precipitated extracellular enzyme was higher than that of the intracellular enzyme (Figure 2b), so that the extracellular enzyme was used for further characterization.



stippled: Supernatant; solid black: Precipitated protein

Figure 1: Protein concentration of the crude extract enzyme precipitated using ammonium sulphate.



the optimum pH for the activity of the AHL-lactonase was at pH 5 and 6 indicated by the highest value of the quorum quenching index relative (Figure 3a). The enzyme also had good stability to the temperature treatments of 40-90 °C for 30 min indicated by the

relatively low decreasing of the quorum quenching index relative values (Figure 3b). The activity of dialyzed AHL-lactonase was higher than those of precipitated enzyme

and crude extract enzyme, while the protein concentration of dialyzed enzyme was lower than that of the precipitated enzyme (Table 2).

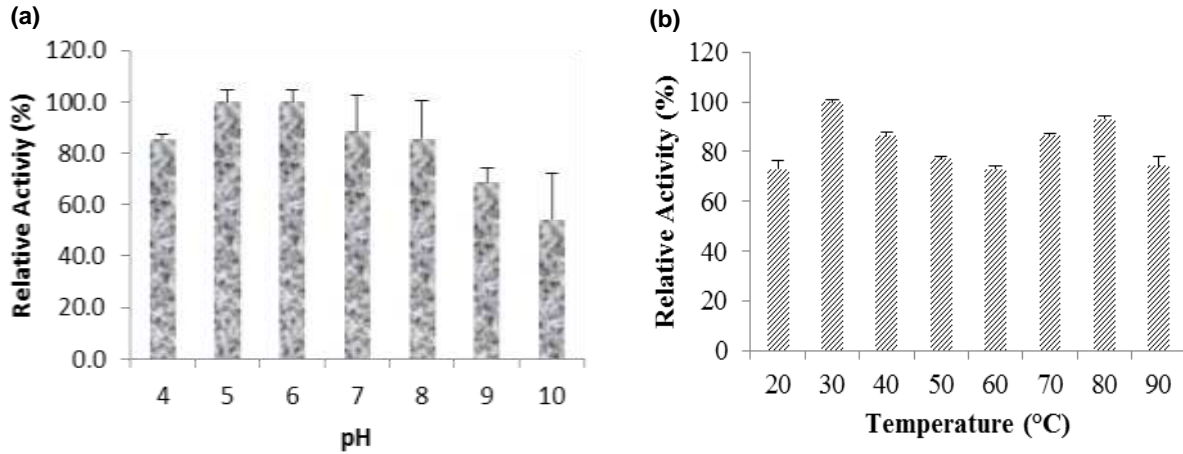


Figure 3: Relative activity of AHL-lactonase at a, various pH and b, temperature treatments.

Table 2: Relative activity of *B. thuringiensis* SGT3g AHL-lactonase during precipitation process.

Precipitation Steps	Volume (mL)	Quorum Quenching Index	Protein Concentration (mg/mL)	Total Protein (mg)	Relative Activity (%)
Crude Extract	200	0.812	0.583	116.6	100
Precipitation	2	0.969	0.916	1.832	103.6
Dialysis	2	1.406	0.3415	0.683	126.4

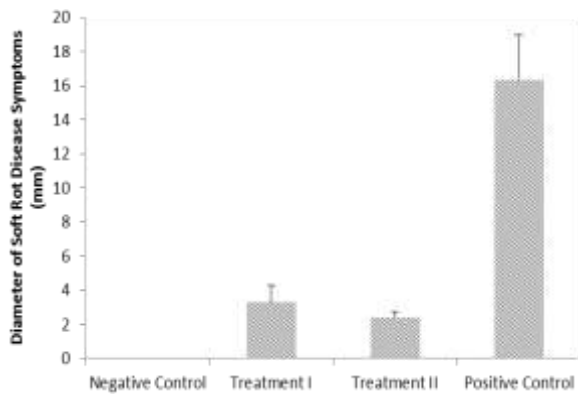


Figure 4: Diameter of soft rot disease symptoms on hybrid orchid *Phalaenopsis* leaves (Treatment II was co-culture of *B. thuringiensis* SGT3g with *D. dadantii*).

In planta inhibition assay of *B. thuringiensis* SGT3g against *D. dadantii*

The results showed that the rot symptom only occurred on positive control leaves after 24 h incubation. The leaves which inoculated with *B. thuringiensis* SGT3g showed less-severe rot symptom than that of the positive control. The rot symptom occurrence on leaves inoculated with *B.*

thuringiensis SGT3g was 50%, while on the positive control was 100% rot symptom occurrence. The diameter of rot symptom on leaves inoculated with *B. thuringiensis* SGT3g was 3.28 mm, while on the positive control was 16.3 mm (Figure 4). However there was reduction of *D. dadantii* cell number on the leaves inoculated with *B. thuringiensis* SGT3g (Table 3). The co-culture treatment showed us the cell number of *D. dadantii* slightly higher than the not co-culture treatment (Treatment I). The soft rot symptoms on co-culture treatment showed lesser than not co-culture method (Figure 4).

Table 3: Comparison of *D. dadantii* cell number on the leaves of hybrid orchid *Phalaenopsis*.

Inoculation treatments	Bacterial cell number of <i>D. dadantii</i> (CFU/mL)
Inoculum	$1.12 \times 10^9 \pm SE 1.49 \times 10^8$
Positive control	$1.01 \times 10^9 \pm SE 2.9 \times 10^7$
Treatment I	$3.96 \times 10^8 \pm SE 3.41 \times 10^7$
Treatment II*	$7.00 \times 10^8 \pm SE 1.75 \times 10^8$

*Treatment II was co-culture method

DISCUSSION

This study showed that *B. thuringiensis* SGT3g produced intracellular and excreted AHL-lactonase enzyme outside the cell as an extracellular enzyme. Some studies reported that AHL-lactonase was produced by *B. thuringiensis* as an intracellular enzyme (Cao *et al.*, 2012; Sakr *et al.*, 2014). AHL-lactonase also produced in extracellular enzyme by *Muricauda olaria* which is not belongs to *Bacillus* group (Tang *et al.*, 2015). In this study indicated that *B. thuringiensis* SGT3g produces both extracellular and intracellular AHL-lactonase. The AHL lactonase could hydrolyze AHL signal of *C. violaceum* indicated by the formation of non-purple zone around paper discs, because of the absence of violacein synthesis. The AHL signal is secreted outside the cell of *C. violaceum* for violacein pigment secretion process (McClellan *et al.*, 1997) which is responsible for the violet color in *C. violaceum* bacterial colony appearance. The other Gram negative bacterium *D. dadantii* also uses AHL signal for expression of virulence factor to degrade cell wall of plant.

The highest precipitated protein concentration of *B. thuringiensis* SGT3g crude extract enzyme of was at 70% (w/v) ammonium sulphate with the highest quorum quenching index value of AHL-lactonase. Most studies reported that AHL-lactonases could be precipitated using ammonium sulphate in range of 30-80% saturation (Seo *et al.*, 2011; Chen *et al.*, 2010; Mukherjee and Prabhune, 2015). The recombinant AHL-lactonase of *Bacillus* sp. B546 expressed on *Pichia pastoris* was successfully precipitated using 80% ammonium sulphate (Chen *et al.*, 2010). Addition of ammonium sulphate up to 70% saturation precipitated *B. thuringiensis* SGT3g AHL-lactonase optimally and increased the enzyme activity up to 1.2 times. However, the increasing of this enzyme activity is lower than that of *Geobacillus caldxylosilyticus* YS-8 AHL-lactonase precipitated using 40-60% ammonium sulphate saturation, which the increasing could reach up to 3.15 times (Seo *et al.*, 2011).

The precipitated AHL-lactonase was purified through dialysis process to eliminate ammonium sulphate salt and other non-protein dissolved matters that their presence will inhibit the binding process of the substrates to the active site of AHL-lactonase, and eventually affecting the enzyme activity (Scopes, 1994). The total protein of AHL-lactonase after dialysis was significantly decreased during dialysis process due to small proteins exited from the membrane. This study uses a 12.4 kDa dialysis membrane, so that proteins smaller than that size will exit from the membrane, while those bigger proteins will remain in the membrane. The molecular weight of the AHL lactonase from *B. thuringiensis* SGT3g was 28.77 kDa based on SDS-PAGE analysis (Asmarani, 2014). Commonly the AHL Lactonase proteins of *AiiA* gene from *Bacillus* have molecular weight in the range of 28 – 31.5 kDa (Chen *et al.*, 2010; Cao *et al.*, 2012). The AHL lactonase of *G. caldxylyticus* YS-8 has molecular weight of 32 kDa (Seo *et al.*, 2011) and AHL lactonase of recombinant *M. olearia* was 31 kDa (Tang *et al.*, 2015).

The optimum activity of AHL-lactonase of *B. thuringiensis* SGT3g was at pH 5 and 6, its mean that the enzyme was active at acidic condition. Tinh *et al.* (2013) reported that the recombinant AHL-lactonase from *B. cereus* had activity in pH range of pH 5-8. The prediction of AHL-lactonase protein produced by *B. thuringiensis* SGT3g was an acidic protein with isoelectric point at pH 4.7 (Asmarani, 2014). Wang *et al.* (2004) also reported that isoelectric point of AHL-lactonase was at pH 4.17. Although acidic condition can trigger re-lactonation to occur in opened AHL-ring (Dong *et al.*, 2001; Chen *et al.*, 2013; Ghani *et al.*, 2014), the present study suggested that AHL-lactonase of *B. thuringiensis* SGT3g had optimum activity at pH 5-6. AHL-lactonase of *Bacillus* sp. AI96 had optimum activity at pH 8, however it still had activity in pH range of 6-9 (Cao *et al.*, 2012). While AHL lactonase of *B. weihenstephanensis* P65 had activity in pH range of pH 6-9 (Sakr *et al.*, 2013).

The highest AHL-lactonase activity was found at 30 °C temperature treatment, however the enzyme still had activities after temperature treatments at 40-90 °C for 30 minutes incubation. This result indicated that the enzyme is resistant to high temperature. AHL lactonase of *B. thuringiensis* had optimum activity at 25-60 °C, and it was still able to show its activity after temperature treatment up to 70 °C (Sakr *et al.*, 2014).

Application of the AHL-lactonase of *B. thuringiensis* SGT3g could decrease soft rot disease symptoms caused by *D. dadantii* on *P. ekanagasaki* leaves (Figure 4). Previously, Dong *et al.* (2004) demonstrated the capability of AHL-lactonase produced by *B. thuringiensis* to inhibit QS process of soft rot disease symptoms on a potato. Role of AHL-lactonase in inhibiting pathogenicity of the soft rot disease bacteria was described clearly using transgenic plants. Transgenic tobacco, *A. konjac*, and cabbage which expressed the inserted *aaiA* gene encoding AHL-lactonase were able to inhibit soft rot disease (Dong *et al.*, 2001; Dong *et al.*, 2002; Ban *et al.*, 2009). However there was reduction of *D. dadantii* cell number on the leaves inoculated with *B. thuringiensis* SGT3g. This reduction cell number of *D. dadantii* might be due to limited nutrient availability and nutrient competition between *D. dadantii* and *B. thuringiensis* SGT3g on the leaves. Besides *B. thuringiensis* is well known as one of the successful environment-friendly biopesticides to control insect pests, based on the results, *B. thuringiensis* SGT3g also has potential application to be used as a biocontrol agent against phytopathogenic bacteria with the quorum quenching mechanism.

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