Biological control of potato tuber soft rot using *N*-acyl-L-homoserine lactone-degrading endophytic bacteria

Nguyen Thanh Ha[1], Tran Quang Minh[2], Pham Xuan Hoi[1], Nguyen Thi Thanh Thuy[3], Naruto Furuya[4] and Hoang Hoa Long[1,*]

[1]Agricultural Genetics Institute, Pham Van Dong, Tu Liem, Hanoi, Vietnam
[2]Soils and Fertilizers Research Institute, Dong Ngac, Tu Liem, Hanoi, Vietnam
[3]Ministry of Agriculture and Rural Development, Ngoc Ha, Ba Dinh, Hanoi, Vietnam
[4]Kyushu University, Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

***Pectobacterium carotovorum* (*Pc*) causing potato tuber soft rot uses *N*-acyl-L-homoserine lactones (AHLs) to control the production of virulence factors via quorum sensing (QS). Some bacteria produce enzymes to inactivate the AHL signals of pathogenic bacteria via a phenomenon known as quorum quenching. One hundred bacterial isolates from potato tubers were screened for AHL-degrading activity using biosensor strain *Chromobacterium violaceum* CV026. Of these isolates, 20 were able to inactivate AHLs from the pathogenic bacteria *in vitro*. Of the 20 isolates, 6 attenuated tissue maceration of potato tubers by *Pc*. Suppression of tuber soft rot was observed even when these isolates were applied 24 h after the pathogen was introduced. Their colonization in tubers was approximately 10^3–10^4 cfu/g tuber, 7 days after inoculation. These isolates were identified as *Bacillus* sp., *Variovorax* sp., *Variovorax paradoxus* and *Agrobacterium tumefaciens*. Four of these isolates showed putative AHL-lactonase activity and provided the most significant protection against *Pc*. Therefore, AHL-degrading endophytic bacteria can be utilized as a novel biocontrol agent of potato tuber soft rot in Vietnam.**

Keywords: AHL, *Pectobacterium carotovorum*, potato tuber soft rot, quorum sensing, quorum quenching.

GRAM-negative bacteria utilize *N*-acyl-L-homoserine lactones (AHLs) as autoinducers to coordinate gene expression in a population density-dependent manner referred to as quorum sensing (QS)[1]. *Pectobacterium* (formerly *Erwinia*), an enterobacterial plant pathogen, causes soft rot diseases in monocot and dicot species[2]. *P. carotovorum* (*Pc*) is the causative agents of soft rot and blackleg in potato and affects plant health during field production and storage[3]. The soft rot pathogens promote disease by producing plant cell wall-degrading enzymes which then facilitate the infection and cause maceration of plant tissue. QS controls not only production of such degrading enzymes, but also regulates production of virulence determinants and secondary metabolites. *Pc* synthesizes AHLs which play an important role in regulating gene expression in the phytopathogen[4].

Pathogenic QS systems are potential targets for anti-virulence strategies as many bacterial pathogens control the expression of virulence factors via QS signalling. The disruption of QS signalling, also called 'quorum quenching' (QQ), has been described in bacteria, fungi, plants, animals, etc.[5–8]. The use of endophytic bacteria living inside plant tissues capable of naturally degrading AHL produced by pathogens has been considered as a suitable approach to interfere with infection[9]. To study this, AHL-degrading bacteria have been isolated from various sources like leaf surface of potato plants[9], tobacco phylosphere[10], rhizosphere[11,12] and soil samples[13].

Endophytic bacteria are referred to as the bacteria that can be detected at a particular moment within the tissues of apparently healthy plant hosts[14] and increase the plant's resistance against pathogens. Endophytic bacteria are actually soil-borne but can also penetrate plant roots, and some strains may move to aerial parts – like xylem, bark and leaves – although bacterial densities in above ground tissues are low compared to root colonizing populations[15]. Endophytic bacteria have been isolated from within the tissues of potato plants, especially from within potato tubers[16]. They were shown not only to inhibit disease development, but also to promote growth and tuberization[17]. The extended and stable relationships between endophytic bacteria and their potato hosts make them an ideal source for selecting candidate biocontrol agents[11,18]. The advantage of using endophytic bacteria in the potato tubersphere for biocontrol would be to eliminate the need to select tubersphere competent bacterial types. Moreover, since they have the ability to reside within the same micro-habitat as their pathogenic counterparts, their efficiency may be relatively greater. However, little is known about the AHL-degrading endophytic bacteria that naturally reside inside potato tubersphere. Therefore, the goal of this study is to identify efficient AHL-degrading

*For correspondence. (e-mail: hoanghoalong@gmail.com)

endophytic bacteria suitable for biological control of potato tuber soft rot in Vietnam.

Materials and methods

Microorganisms and culture conditions

Endophytic bacteria were isolated from 27 tubers of the potato plants (*Solanum tuberosum* L. cv KT3) at the maturation stage collected from different potato growing areas in Hanoi, Vietnam and its adjacent provinces. *Pectobacterium carotovorum* P2.5.1 (*Pc*) isolated from rotten potato tuber in Vietnam was used as potato soft rot pathogen[19]. *P. carotovorum* subsp. *carotovorum* ATCC15713^T (*Pcc*) was used as a source of naturally producing AHL molecules (3-oxo-C6-HSL) with short-acyl chain[4]. *Burkholderia glumae* MAFF301169^T (*Bg*) was used as source of naturally producing AHL molecules (C8-HSL) with long-acyl chain[20]. *Chromobacterium violaceum* CV026 (ref. 21) and *C. violaceum* VIR07 (ref. 22) were used as the indicator strains for AHL detection. The media used were Luria-Bertani (LB) and Yeast Peptone Dextrose Agar (YPDA, Sigma, Steinheim, Germany). All synthetic *N*-hexanoyl-L-homoserine lactone (C6-HSL) were purchased from Cayman Chemical (Cayman Chemical Company, Ann Arbor, MI, USA).

Isolation of endophytic bacteria

Endophytic bacteria were isolated as described by Long *et al.*[23]. Potato tubers were surface-sterilized using 70% ethanol for 1 min, sodium hypochlorite solution (3% available Cl^-) for 5 min and rinsed three times in sterilized distilled water. The final wash was plated on 0.5 × YPDA and the plates were incubated at 30°C for 2–10 days to verify the disinfection procedure. A piece of sterilized potato tuber (5 × 5 × 5 mm) containing eyes or lenticels was titrated in distilled water and the appropriate dilutions were plated onto 0.5 × YPDA and incubated at 30°C for 2–10 days. Typical colonies were selected, cultured and preserved for long-term use.

Bioassay for degrading natural AHLs

Natural AHL producers (*Pcc/Bg*), biosensor strains CV026/VIR07 and endophytic bacterial isolates were parallel-streaked as homogenous lines on LB agar medium. After incubation at 28°C for 24 h, no appearance of purple pigment in CV026/VIR07 revealed the degradation of natural AHLs by bacterial isolates and appearance of purple pigment in CV026/VIR07 indicated no degradation of natural AHLs as well as production of AHLs by the pathogens. Six bacterial isolates that showed stable and strong degradation activity of natural AHLs produced by *Pcc* were selected for further studies.

Identification of selected endophytic bacterial isolates

Total genomic DNA of the six selected bacterial isolates mentioned above was extracted from 1-day-old cultures. PCR procedure was carried out as described by Long *et al.*[24] using primers F27 (5′-AGAGTTTATCMTGGCTCAG-3′) and R1492 (5′-GRTACCTTGTTACGACTT-3′)[25]. A negative control (PCR mixture without DNA template) was included in all PCR reactions. The resulting PCR products were sequenced by a Big Dye Terminator and ABI Prism 3700 Genetic Analyzer (Macrogen, World Meridian Venture Centre, Korea) and at least 600 bp were subjected to the BLAST analysis.

In vitro antibacterial activity of bacterial isolates

To detect the antibacterial activity of the selected six isolates against *Pc*, the chloroform vapour assay[26,27] by which the bacterial cultures were killed by chloroform vapour was used. A fresh culture (1–2 days) of an endophytic bacterial isolate from YPDA slant was transferred to YPDA plate. The plates were then incubated at 30°C for 2–3 days. After the bacteria formed colonies several mm in diameter, the plate was turned upside down. A sheet of filter paper was placed in the petri dish lid and 2 ml of chloroform was added to it and kept at room temperature for 3 h. After complete evaporation of chloroform, the *Pc* P2.5.1 bacterial suspension (conc. ca. 10^8 cfu/ml, 0.5 ml) was mixed with 5 ml of 0.5% plain agar medium (melted and kept at 50°C) and overlaid on the plate and incubated at 30°C for 2 days. If an inhibition zone appeared, its width was measured to evaluate the activity or productivity of antibacterial substances. A bacterial isolate that is known to exhibit the *in vitro* antibacterial activity against *Pc* was used as a control. The experiment was conducted in duplicate.

Attenuation of potato tissue maceration caused by P. carotovorum

Six AHL-degrading bacterial isolates (AGI1.6, AGI1.13, AGI2.7, AGI2.8, AGI6.4 and AGI7.1) were subjected to test the ability to attenuate tissue maceration on potato tuber by *Pc* P2.5.1 following Molina *et al.*[28] with some modifications. An AHL non-degrading isolate, AGI7.9 and sterile distilled water (SDW) were used as controls. Briefly, potato tubers were washed and cut into 0.7 cm thick slices. Approximately 5 mm × 5 mm wells on slices were made using a cork borer. The slices were surface sterilized by incubation in 5% sodium hypochlorite for

5 min and then rinsed three times with sterilized water. Each slice was placed in a single petri dish. The wells were filled with 50 µl of a mixture of *Pc* P2.5.1 suspension at a density of 10^8 cfu/ml and with AHL degrading bacterial isolates at a density of 10^7 cfu/ml. Also, in another set of experiments, AHL-degrading bacteria were added either 1 day before or 1 day after the pathogen. Wells of the control slices were separately inoculated with *Pc* P2.5.1 suspension (10^8 cfu/ml) or the tested isolate (10^7 cfu/ml). Six replications were conducted for each treatment. The experiment was repeated twice. After inoculation, the potato tuber slices were incubated at 28°C for 24 h. The amount of rot tissues was quantified by comparing the weight difference of potato slices before and after washing the potato slices under running tap water. Treatment means from each independent experiment were compared and analysed with Fisher PLSD's test ($P < 0.05$).

Characterization of AHL-degrading activity

AHL lactonase degrades AHLs by hydrolysing the lactone ring that can be re-circularized in acidic solutions[8]. We tested the presence of putative lactonase activity using the method described by Morohoshi *et al.*[9] with some modifications. Briefly, 90 µl of the supernatant of endophytic bacteria grown in 0.5 × YPD broth supplemented with 10 µM of C6-HSL for 6 h was mixed with 10 µl of 1N HCl. After incubation for 48 h at 4°C, 20 µl of 1M phosphate buffer (pH 7.0) was added for neutralization. The restored C6-HSL was detected by CV026 biosensor. Two independent experiments were carried out.

Bioassay for colonization of bacterial endophytes in potato tuber

For the *in vitro* colonization assays, potato tubers were washed and surface-sterilized as described above. The sterilized potato tubers were dipped in the selected bacterial suspension at a density of 10^7 cfu/ml for 1 h. Each tuber was placed in a dish at room temperature. One week after inoculation, bacterial re-isolation was carried out as described above. Two independent experiments were carried out.

Results

Isolation and screening of natural AHL-degrading endophytic bacteria

One hundred bacterial isolates were collected from 27 potato tubers and screened for the pathogen-excreted AHL-degrading activity using biosensor strains CV026 and VIR07. Twenty isolates were able to degrade AHLs of *Pcc* and twelve of these isolates were capable of degrading *Pcc*'s short-chain AHLs (detected by the biosensor strain CV026) and six of these isolates were able to degrade *Bg*'s long-chain AHLs (detected by the biosensor strain VIR07). Only the short-chain AHL degrading bacterial isolates were selected for further tests (Figure 1). An AHL non-degrading isolate AGI7.9 was used as negative control when needed.

Identification of AHL-degrading bacterial endophytes

Six isolates (AGI1.6, AGI1.13, AGI2.7, AGI2.8, AGI6.4 and AGI7.1) consistently exhibited degradation activity of natural AHLs produced by *Pcc* and were identified based on 16S rRNA sequencing. These isolates showed a high similarity with *Bacillus* sp. (100%), *Variovorax* sp. (100%), *Variovorax paradoxus* (99%) and *Agrobacterium tumefaciens* (100%). Their nucleotide sequences were deposited in GenBank of NCBI under accession numbers KR080867–KR080872 (Table 1).

Biocontrol of potato tuber soft rot using AHL-degrading bacteria

The six selected AHL-degrading bacterial isolates (AGI1.6, AGI1.13, AGI2.7, AGI2.8, AGI6.4 and AGI7.1) were tested for their ability to control bacterial soft rot mediated by AHL signals. All six isolates provided a significant reduction in tissue rot compared to the AHL non-degrading isolate (Fisher's PLSD test; $P < 0.05$) and the pathogen alone when they were co-inoculated with *Pc* P2.5.1 in potato tuber slices (Figure 2 *a* and *d*). To evaluate if the biocontrol activity of these AHL-degrading bacteria on potato soft rot caused by *Pc* P2.5.1 was

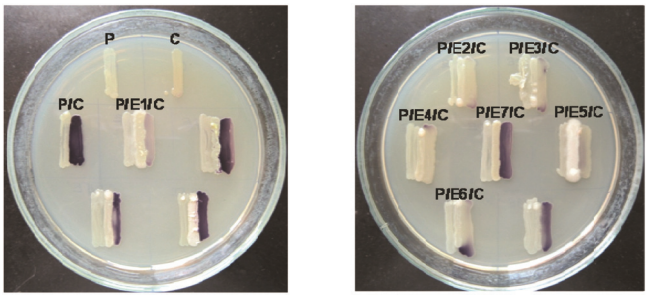

Figure 1. Natural AHL production by *P. carotovorum* subsp. *carotovorum* ATCC15713^T (*Pcc*) and detection of natural AHL-degradation activity of bacterial isolates. *P. carotovorum* subsp. *carotovorum* ATCC15713^T (P), tester strain (E) and *C. violaceum* CV026 (C) were streaked as homogenous lines on LB agar medium. No appearance of purple pigment in CV026 revealed the degradation of natural AHLs by bacterial isolates and appearance of purple pigment in CV026 indicated no degradation of natural AHLs as well as production of AHLs by *Pcc*. Seven bacterial isolates are numbered from E1 to E7 in which E1–E6 are encoded as AGI1.6, AGI1.13, AGI2.7, AGI2.8, AGI6.4 and AGI7.1 (AHL-degraders) and E7 as AGI7.9 (AHL non-degrader).

Table 1. Natural AHL-degrading activity of bacterial isolates from potato tuber

Bacterial isolates	Bacterial identity*	GenBank Acc. No.	AHL-degrading ability	
			Pcc_AHL	Bg_AHL
AGI1.6	*Bacillus* sp.	KR080867	+	–
AGI1.13	*Variovorax* sp.	KR080868	+	–
AGI2.7	*Variovorax paradoxus*	KR080869	+	–
AGI2.8	*Agrobacterium tumefaciens*	KR080870	+	–
AGI6.4	*Bacillus* sp.	KR080871	+	±
AGI7.1	*Bacillus* sp.	KR080872	+	–

–, Negative; ±, slight colour decrease; +, positive; Pcc_AHL, *Pectobacterium carotovorum* subsp. *carotovorum* ATCC15713^T AHL; Bg_AHL, *Burkholderia glumae* MAFF301169 AHL.
*The bacterial isolates were identified via 16S rRNA sequencing with homology search ≥99% by BLAST.

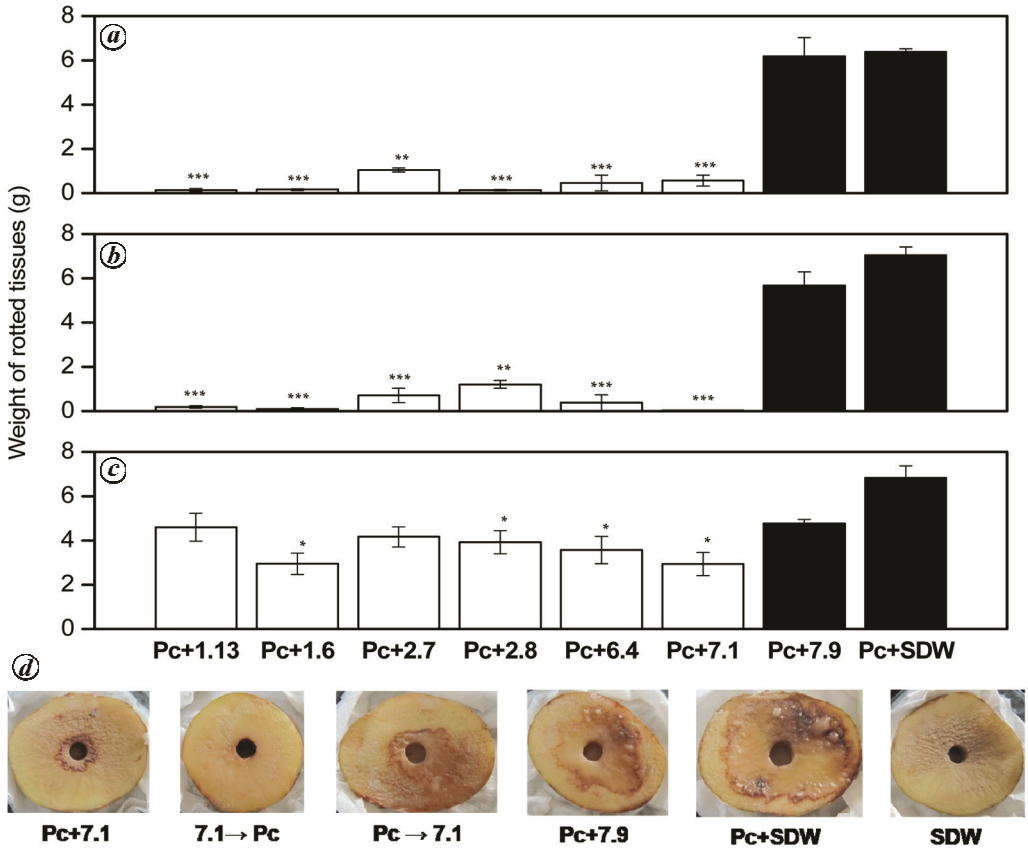

Figure 2. Biocontrol activity of AHL-degrading bacteria against potato tuber soft rot caused by *P. carotovorum* (*Pc*). Using potato tuber slice assay, selected isolates were co-inoculated with *Pc* (***a***) or inoculated into wells 1 day before (***b***) or 1 day after (***c***) the pathogen to demonstrate preventive or curative suppression. Treatments included the pathogen plus AHL-degrader isolates or the AHL non-degrading control AGI7.9 and sterilized distilled water. Potato tuber slices were incubated for 2 days following inoculation with *Pc*. Weight of rotted tissue was quantified. Potato tuber slice assay of isolate AGI7.1 is presented in ***d*** in which Pc + 7.1; 7.1 → Pc and Pc → 7.1 describe the inoculation order as in ***a***, ***b*** and ***c*** respectively. Values represent mean of six treatment replications. Bars indicate standard error of the mean. Asterisks indicate significant differences between the treatments and the controls (Fisher's PLSD test; $P < 0.05$ (*); $P < 0.001$ (**); and $P < 0.0001$ (***)). 1.13, 1.6, 2.7, 2.8, 6.4, 7.1 and 7.9 are encoded for AGI1.13, AGI1.6, AGI2.7, AGI2.8, AGI6.4, AGI7.1 and AGI7.9 respectively.

preventive and/or curative, potato tuber inoculation with the pathogen and the six selected isolates was staggered. When the AHL-degrading bacteria were inoculated one day before the pathogen, the rot was almost completely prevented and a significant reduction in tissue rot was observed (Fisher's PLSD test; $P < 0.05$) (Figure 2 *b* and *d*). When the AHL-degrading bacteria were applied a day after the pathogen, we observed rotting, but this rotting was stopped when the AHL-degrading bacteria were inoculated, resulting in significantly less total rot in the

four treatments of AGI1.6, AGI2.8, AGI6.4 and AGI7.1 (Fisher's PLSD test; $P < 0.05$) compared to the controls using AHL-non-degrading isolate or pathogen alone (Figure 2 *c* and *d*). This demonstrates both preventive and curative biocontrol activity of the AHL-degrading bacterial isolates.

In this experiment, we studied the *in vitro* antibacterial activity of the six AHL-degrading bacterial isolates against *Pc* P2.5.1 in order to compare the relative efficacy of AHL degradation versus antibiotic production as biocontrol mechanisms. However, none of the selected isolates exhibits the antibacterial activity against *Pc*.

Identification of the AHL degradation pathway

Lactonase activity converts AHLs to their cognate derivative, i.e. their ring-open form that is not an active QS signal molecule. We studied whether the AHL-degrading activity was caused by lactonolysis. To do so, the supernatant of the selected six bacterial isolates was mixed with HCl and phosphate buffer (pH 7.0) was added for neutralization. The restored C6-HSL was detected by CV026 biosensor. All the selected bacterial isolates could degrade C6-HSL after 6 h. However, only the degraded C6-HSL in the samples of AGI1.6, AGI1.13, AGI2.7 and AGI2.8 was restored by acidification (Figure 3), while those of other two isolates were not. In addition, the C6-HSL restoration ability of the four isolates was highly variable in which the isolates AGI2.7 and AGI2.8 showed the strongest activity. These results indicated that the putative AHL lactonase activities were present in the above four isolates.

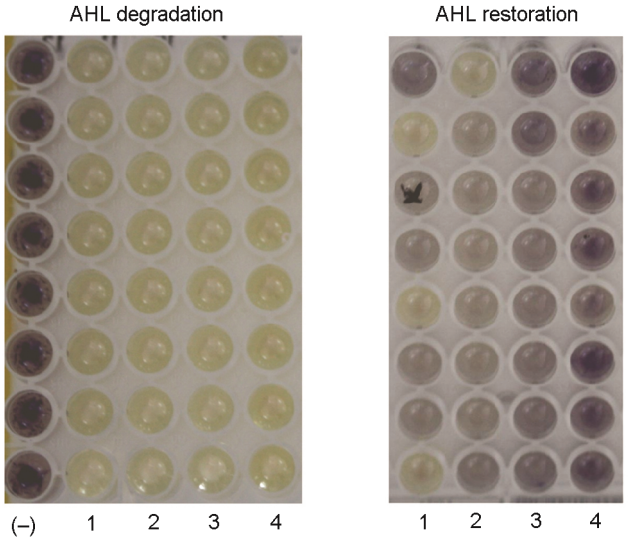

Figure 3. AHL degradation and restoration by bacterial isolates. The selected AHL-degrading bacterial isolates were incubated in YPD medium containing 10 µM C6-HSL for 6 h. The remaining C6-HSL was visualized by *C. violaceum* CV026 in a 96 well plate (AHL degradation). The degraded C6-HSL was recircularized by acidification and visualized by CV026 (AHL restoration). (–) represent the control without bacterial inoculation. Numbers 1–4 encoded bacterial isolates AGI1.6, AGI1.13, AGI2.7 and AGI2.8 respectively.

Colonization of AHL-degrading bacteria and P. carotovorum in potato tuber

Colonization of the six AHL-degrading bacterial isolates and *Pc* inside potato tuber tissues was carried out as described by Long *et al.*[23]. One week after inoculation, most of these isolates were colonized in the tuber at 10^3–10^4 cfu/g fresh tuber weight (Table 2). Among these, the isolates AGI1.6 and AGI6.4 were found at the highest density of 4.6×10^4 and 3.2×10^4 cfu/g fresh tuber weight respectively. *Pc* was colonized at the density of approximately 10^3 cfu/g fresh tuber weight.

Discussion

The microenvironment of potato tubersphere is where the interactions between the soft rot pathogen and beneficial endophytes take place. Wounds and natural openings such as stem ends, lenticels and eyes are common points of entry for pectolytic bacteria[29], whereby they maintain the cell growth by the AHL-mediated QS mechanism. Degradation of the QS signalling molecules will suppress pathogen virulence and reduce disease severity[5]. Since colonizing in the same micro-habitat as the pathogen, AHL-degrading endophytic bacteria are considered a promising source for potential biocontrol agents against tuber soft rot disease caused by *P. carotovorum*.

Here we report on the isolation and identification of AHL-degrading endophytic bacteria from potato tubers, able to significantly inhibit the tissue maceration caused by *Pc*. We also demonstrated that these bacterial isolates were able to interfere with the pathogen's QS system independent of the growth inhibition mechanism. We showed that potato tuber peel layers accommodated a number of endophytic bacteria which inhibited the soil-borne plant pathogens, *Fusarium sambucinum*, *F. avenaceum*, *F. oxysporum* and *Phytophthora infestans*[17]. Sturz

Table 2. Bacterial colonization in potato tuber one week after inoculation

Bacterial isolates	Bacterial endophyte density (cfu/g FW)	Pc density (cfu/g FW)
AGI1.6	4.6×10^4	2.5×10^3
AGI1.13	1.6×10^3	4.2×10^3
AGI2.7	3.0×10^3	2.1×10^3
AGI2.8	2.4×10^3	2.7×10^3
AGI6.4	3.2×10^4	1.5×10^3
AGI7.1	7.2×10^3	3.6×10^3

cfu, Colony forming unit; FW, Fresh weight.

and Matheson[30] also showed that some endophytic bacteria within potato tubers promote resistance to bacterial soft rot *E. carotovora* var. *atroseptica*.

Several types of enzymes which are able to degrade AHLs include AHL-lactonase, AHL-acylase, AHL-deaminase, and paraoxonases[8,31]. AHL-lactonase which breaks down AHLs by hydrolysing the homoserine lactone ring is not dependent on the length and substitutions in the AHL signal molecule[32,33]. Of the six selected AHL-degrading bacterial isolates, four were able to restore the lactone ring of C6-HSL under the acidic pH conditions. However, the two isolates unable to restore C6-HSL exhibited strong biocontrol activity against *Pc*. This suggests that either their QQ activity via non-enzymatic type or other mechanisms might be involved in their biocontrol activity against potato soft rot. The underlying mechanisms of these isolates will be studied in the future.

Previous studies have shown that *Bacillus*, *Streptomyces*, *Arthrobacter*, *Pseudomonas*, *Mesorhizobium*, *Ochrobactrum*, *Chryseobacterium* and *Microbacterium* were able to degrade AHLs[5,9,11,27,34,35]. However, to the best of our knowledge, there is only one report on an AHL degrading strain from potato, *V. paradoxus* S110, which was isolated from the interior of a plant grown at a farm upstate New York (USA)[36]. Our *V. paradoxus* AGI2.7, that was isolated from the interior of potato tubers, also possesses this activity and it was found to be the most potent isolate for AHL degradation among the selected species. It also effectively reduced plant tissue maceration caused by *Pc*. It has been reported that *V. paradoxus*, a metabolically diverse, aerobic bacterium, involves in beneficial interactions with other bacteria and plants[37,38].

Our studies also demonstrated that natural AHL-degrading bacterial endophytes have curative as well as preventive biocontrol activity. We found that even when the pathogen was inoculated one day ahead to start inducing disease symptoms, the subsequent application of an AHL-degrading bacterial isolate suppressed further disease development. This is consistent with a report on the bacterial soft rot curative activity of a transgenic AHL degrader, *Pseudomonas fluorescens* P3/pME6863, which was even curative when inoculated two days after the pathogen[27]. In other reports, it was also found that AHL degrading *Bacillus* isolates and *Lysinibacillus* sp. Gs50 attenuated symptoms of *Pcc* soft rot in both curative and preventive control studies[39,40].

We found that the selected endophytic bacterial isolates could colonize the potato tuber tissues at densities between 10^3 and 10^4 cfu/g fresh weight, one week after inoculation. Sturz *et al.*[17] also reported that bacterial population density was variable from the outermost peel (periderm) to the innermost peel location. However, their density in the peel layer was generally about 10^3cfu/g fresh weight of peel material. Notably, the population of *Pc* remained about 10^3 cfu/g fresh weight indicating that the pathogen growth inhibition was not observed in the co-inoculated potato tubers. This finding is consistent with that of Molina *et al.*[27] who found out that the wild-type strain *Bacillus* sp. A24, which did not inhibit the growth of *E. carotovora*, was able to control soft rot disease of potato tubers.

In conclusion, we have identified several potential biological control agents of potato tuber soft rot belonging to the class of the AHL-degrading endophytic bacteria without antagonism activity against the pathogen *Pc*. The capability of endophytic bacteria to degrade major signalling molecules of *Pcc*, i.e. AHLs, suggests that we have found a novel source for AHL lactonase. The study reinforces the notion that using QS quenchers to obtain biological control of plant pathogens is a promising strategy to improve crop protection.

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ACKNOWLEDGEMENTS. This research is funded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106.03-2012.61. We thank Dr Merijn R. Kant (University of Amsterdam) for his excellent comments.

Received 1 September 2016; revised accepted 3 August 2018

doi: 10.18520/cs/v115/i10/1921-1927