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RESEARCH ARTICLE

Pseudomonas as a frequent and important quorum quenching bacterium with biocontrol capability against many phytopathogens

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ABSTRACT

The aim of the present study was to isolate a variety of quorum quenching bacteria (QB) from the rhizosphere and phyllosphere of three agricultural plants using minimal medium (MM)- and non-minimal medium (NM)-based methods. The members of the *Pseudomonas* genus constituted the most abundant QB genus, particularly in the rhizospheres of all plant samples and showed the highest quorum quenching (QQ) activity according to a screening assay using a biosensor and 3-oxo-C6-HSL (as an important quorum sensing signal in many phytopathogenic bacteria). In addition, QQ-*Pseudomonas* were recognised as versatile biocontrol agents against non-bacterial and bacterial plant pathogens, such as *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc). Three types of quenching activities, including intracellular and extracellular enzymatic and non-enzymatic activities, were observed in QQ-*Pseudomonas*. *Pseudomonas* strains, particularly NM-isolated strains with extracellular activity, are the strongest QQ-based biocontrol agents.

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Quorum quenching bacteria; rhizosphere; phyllosphere; *Pseudomonas*

1. Introduction

Quorum sensing (QS), as a form of intercellular signalling, enables bacterial populations to coordinate important biological functions, including luminescence, motility, aggregation, plasmid conjugal transfer, biosynthesis of antibiotics, biofilm, symbiosis, and virulence, in response to fluctuations in the cell population density (Swift et al., 2001; Waters & Bassler, 2005). There are different types of QS operating signal molecules; N-acyl-L-homoserine lactones (AHLs) are the most common QS signal observed in various Gram-negative bacteria. Although all AHLs have a homoserine lactone ring moiety in common, the acyl side chains of different AHLs can vary in length, degree of substitution, and saturation (Dong, Gusti, Zhang, Xu, & Zhang, 2002). AHL-based QS molecules regulate the expression of virulence-related genes in Gram-negative phytobacteria (Coenye, 2010; De Kievit & Iglewski, 2000; Põllumaa, Alamäe, & Mäe, 2012; Von Bodman, Majerczak, & Coplin, 1998; Williams, Winzer, Chan, & Camara, 2007). Amongst all types of AHLs, 3-oxo-C6-HSL is one of the most frequent agents in many strains of plant pathogenic bacteria, such as *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc), *Erwinia amylovora* (Ea),

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Pantoea stewartii, and others (Barnard & Salmond, 2007; Põllumaa et al., 2012; Von Bodman et al., 1998). The biocontrol of an agent reduces the incidence and severity of plant diseases, particularly using beneficial bacteria. In many biocontrol systems, one or more antibiotics play roles in disease suppression (Handelsman & Stabb, 1996; Pal & Gardener, 2006). Antibiotics or antibacterial agents kill or inhibit bacterial growth through interference with essential functions and the imposition of selective pressures that result in antibiotic-resistant microbial pathogens. Therefore, the interruption of QS signalling, known as quorum quenching (QQ), has been suggested as a promising approach to reduce the expression of virulence factors depending on QS regulation without the risk of antibiotic resistance (Dong, Wang, & Zhang, 2007; Dong, Zhang, Xu, & Zhang, 2004; Hentzer & Givskov, 2003). There are quorum quenching bacteria (QBs) with or without application against plant pathogenic bacteria. Various mechanisms have been reportedly involved in the QQ capacity in bacteria, including intracellular and extracellular enzymatic QQ activities (Cheong et al., 2013), for some enzymes, such as lactonase and acylase, in *Bacillus* and *Pseudomonas* species, respectively (Liu et al., 2008; Sio et al., 2006), or non-enzymatic activities, such as the generation of phenethylamide metabolites in *Halobacillus salinus* (Teasdale, Liu, Wallace, Akhlaghi, & Rowley, 2009). Several studies have reported QBs with biocontrol activity (Helman & Chernin, 2015; Ma, Lv, Zhuang, & Zhuang, 2013). Accordingly, several QBs have been used to reduce the virulence factors of plant pathogenic bacteria. For example, *Rhodococcus erythropolis* (Uroz et al., 2003) and *Bacillus* significantly reduced the pathogenicity of Pcc and Ea, respectively (Hanano, Harba, Al-Ali, & Ammouneh, 2014). In addition, some QBs might be equipped with biocontrol activity against non-bacterial phytopathogens, such as *Lysobacter enzymogenes* against *P. carotovorum* and some fungi (Qian et al., 2010). QBs have been isolated from various environments, including rhizospheres and phyllospheres that form nutrient-rich niches for bacterial populations, and many types of bacteria in these niches, including QBs, belong to various prokaryotic taxa (Dulla, 2007). The distinct genera of QBs have been repeatedly isolated from these niches in several studies (Chan, Wong, Yin, Sam, & Koh, 2010; Chong, 2012; Chong et al., 2012). However, no comprehensive study has been conducted on the frequency, biocontrol efficacy, and effect of the QQ mechanisms of these QBs on biocontrol capacity. Therefore, the aim of the present study was to identify the most frequent and strongest biocontrol QQ-based bacterial genera in the rhizospheres and phyllospheres of the three least studied agricultural plants (saffron, fig, and pomegranate) in Iran. To this end, populations of QBs were isolated from rhizospheres and phyllospheres and subsequently screened to identify agents with considerable QQ-based biocontrol activity against 3-oxo-C6-HSL-harbouring pathogenic bacteria, such as Pcc, and other pathogenic bacteria with different AHLs. Furthermore, the QQ mechanisms of these bacteria are discussed to identify additional potent mechanisms of biocontrol against phytopathogenic bacteria and to determine whether these agents possess non-QQ-based biocontrol capacities in the selected QBs.

2. Materials and methods

2.1. Microbial strains and sampling

The microbial strains used in the present study included Pcc with 3-oxo-C6-AHL as the main QS-signal (Pcc6), *Pectobacterium* (Pc8) with 3-oxo-C8-AHL as the main QS-signal,

Chromobacterium violaceum (CV026), *Pseudomonas fluorescens* (without AHL degradation activity), 2 strains of *E. amylovora* (Ea1 with 3-oxo-C6-AHL and Ea2 with one type of 3-oxo-AHL) and *Fusarium solani*. All strains were obtained from the culture collection of Ferdowsi University of Mashhad, Iran. Fifteen samples were collected from the rhizospheric and phyllospheric regions of three plants (saffron, fig, and pomegranate) from different locations, and the samples from each plant were uniformly mixed together (Figure 1).

2.2. Preparation of the bacterial source

For each plant, a master sample was prepared by mixing 10 g of roots and adhesion soil as previously described (Christiaen, Brackman, Nelis, & Coenye, 2011). Similarly, for the phyllospheric master samples, 100 g of the phyllospheric samples was pooled using a previously described method (Morohoshi, Someya, & Ikeda, 2009). Each of the rhizospheric and phyllospheric master samples was divided into 10 samples and used for QB isolation using two methods (five samples for each method; both methods are described in Figure 1).

2.3. Isolation of bacterial strains from the plant's rhizosphere and phyllosphere

The first method was a minimal medium-based method (MM method), according to Christiaen et al. (2011). Two hundred microliters of the medium containing 50 µl of

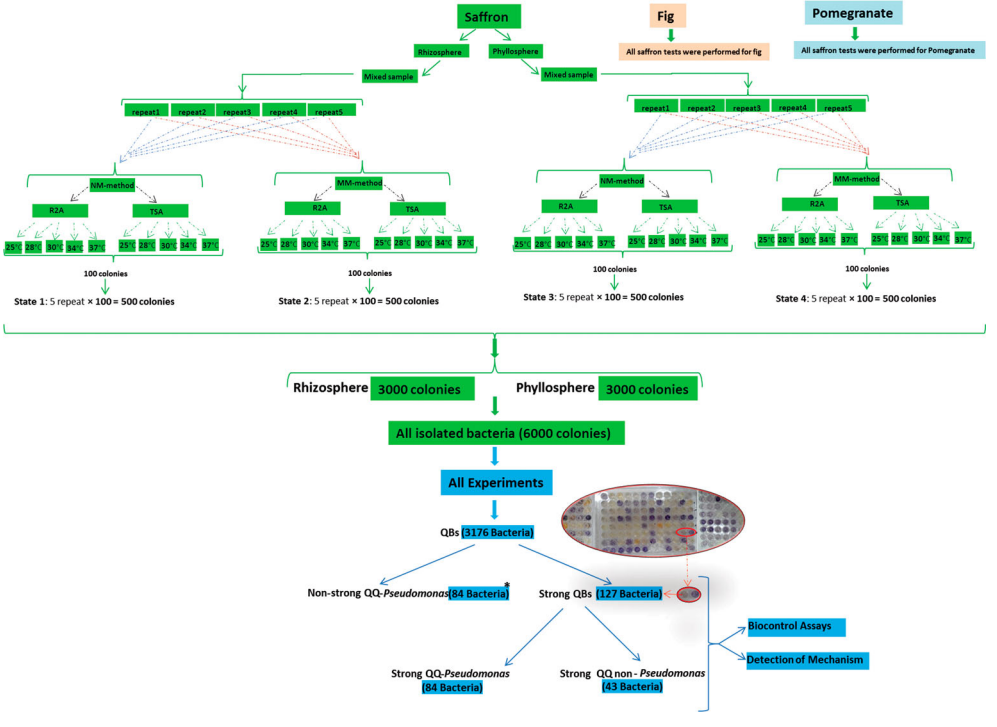

Figure 1. Study procedure at a glance. Green boxes (procedures), blue boxes (isolate selection and some assays). *See Table 3. State 5 to state 12 for the other two plants, fig and pomegranate, were the same as described in saffron tests; thus, these states are not shown in the figure; each state contained 500 colonies (see Table 1, column 1).

3-oxo-C6-HSL (2 mM, Sigma-Aldrich) was passed through a micro-filter (0.22 µm) and added to an equal volume of bacterial solution, followed by incubation at different temperatures (25, 28, 30, 34, and 37°C) for three days. Subsequently, 100 µl of the solution was added to 1 ml of the fresh medium, and the resulting compound was incubated at 25–37°C for three days. This process was repeated three times. The final enrichment was cultured on plates containing Reasoner's 2A agar (R2A) and trypticase soy agar (TSA) media at different temperatures. A total of 500 rhizospheric bacterial colonies were selected for each plant, temperature, and medium (Figure 1). In the second method, a non-minimal medium-based method (NM method), the diluted rhizospheric and phyllospheric samples in the saline buffer were streaked equally onto R2A and TSA media and subsequently incubated at 25–37°C. Bacterial selection was performed as in the first method (Table 1 and Figure 1). Antifungal antibiotics were used in medium cultures for both methods.

2.4. Selection of frequent QQ bacteria

The Gram test was performed for all bacteria. In addition, fluorescence tests were conducted on two iron-limited media for fluorescent *Pseudomonas* screening in casamino acid (CAA) (De Vos et al., 1997) (Figure 3(B)) and King's B (King, Ward, & Raney, 1954) media. Subsequently, an AHL inactivation assay was performed on 96-cell plates as previously described (Morohoshi et al., 2009). The QQ was evaluated using a *C. violaceum* CV026 biosensor (McClean et al., 1997). The non-inoculated medium was mixed with water or AHLs as negative (0% QS activity) or positive controls (100% QS activity), respectively. A second negative control was the *Pseudomonas fluorescent* strain (unable to degrade any AHL molecules). For further confirmation, AHL inactivation assays were randomly repeated for some isolates in 24-well cell cultures (larger volume, Figure 3(A)). After violacein extraction, the coloured solutions were measured at 585 nm using a microplate reader. The isolates with a lighter purple colour compared to the controls were assumed to be QBs. The AHL inactivation assays were repeated for QBs in 0.3 µg/µl 3-oxo-C6-HSL, with eight replications. The isolates inducing the highest reduction in violacein with a significant difference (approximately 5% more than other QB isolates) were selected as strong QBs. In addition, to determine whether these QBs play a role in the inactivation of other AHLs, the strongest QBs screened using O-C6 (and all QBs isolated using the two states Fig-NM method-Rhizosphere and Fig-MM method-Rhizosphere; Table 1 and Figure 1) were subjected to additional screening using the O-C8-biosensor assay.

2.5. Verification of the strongest QBs selection

Eighty-four non-strong QBs were randomly selected to compare their rates of QQ activity with the 84 strong QBs (Table 2).

2.6. Antibacterial activity

The antibacterial activity of QBs was assessed after spotting onto the surface of M9 medium plates using a previously described method (Sammer et al., 2009) and plates

Table 1. Percentages of isolates in the rhizospheres and phyllospheres of the three plants. R: rhizosphere and P: phyllosphere, ND: Not determined (numbers were rounded).

1-States	2-Isolates in all	3-*Pseudomonas*	4-%QBs: Oc6-HSL	5-%QQ-*Pseudomonas*	6-%QQ-*Pseudomonas*	7-%QBs-Oc6-HSL and Oc8-HSL	8-%QQ-*Pseudomonas:* Oc6-HSL and Oc8-HSL	9-%*Pseudomonas:* antibacterial activity
1-Saffron-NM method-R	500 (5 *100)	16.8 d	8.8 c	44 bc	22.7 b	95.78	100	5.9
2-Fig-NM method-R	500 (5 *100)	25.4 c	11.2 c	64.4 a	29.75 b	ND	ND	8.3
3-Pomegranate-NM method-R	500 (5 *100)	30 c	8.8 c	53.3 ab	16 b	ND	ND	3.4
4-Saffron-NM method-p	500 (5 *100)	18.6 d	9.2 c	32.6 cd	17.2 b	ND	ND	2.1
5-Fig-NM method-p	500 (5 *100)	17.6 d	8.2 c	51.9 ab	25.4 b	ND	ND	2.3
6-Pomegranate-NM method-p	500 (5 *100)	8.8 e	8.6 c	13.1 e	12.9 b	ND	ND	2
7-Saffron-MM method-R	500 (5 *100)	46 b	96.2 ab	45.4 bc	95.2 a	99.17	99.56	4.3
8-Fig-MM method-R	500 (5 *100)	54.4 a	94.6 b	54.4 ab	94.6 a	ND	ND	3.7
9-Pomegranate- MM method-R	500 (5 *100)	53 a	97.6 ab	51 abc	94.2 a	ND	ND	3.8
10-Saffron-MM method-p	500 (5 *100)	53.8 a	97.8 ab	52.6 ab	95.7 a	ND	ND	3.3
11-Fig-MM method-p	500 (5 *100)	51.6 a	98.6 a	50.5 abc	96.6 a	ND	ND	1.9
12-Pomegranate-MM method-p	500 (5 *100)	13.8 de	95.6 ab	13.8 de	95.8 a	ND	ND	3.2

Note: Some important results of the statistical analyses were shown. The same letters (a,b,c, …) in a column are not significantly different (Tukey at $P \leq .05$). There are significant differences between some states in columns 3, 4, and 5. There are not significant differences between all states in columns 7, 8, and 9. All analyses and results are not shown.

1-States, 2-Sum of isolates in all samples, 3-Average %*Pseudomonas* amongst all bacteria, 4-Average %QBs with activity against OC6-HSL, 5-Average %QQ-*Pseudomonas* amongst all QBs with activity against Oc6-HSL, 6-Average %QQ-*Pseudomonas* amongst all *Pseudomonas* with activity against OC6-HSL, 7-Average %QBs with activity against OC6-HSL and OC8-HSL, 8-Average % QQ-*Pseudomonas* with activities against OC6-HSL and OC8-HSL, 9-Average %*Pseudomonas* amongst all *Pseudomonas* with antibacterial activity against Pcc6. ND: not determined.

Table 2. Statistical comparisons to verify the biosensor assay results.

Comparisons	Number of isolates	
Between strong ps and strong nps (Totally 127 isolates)	84 ps[a]	43 nps
Between strong ps and non-strong ps (Totally 168 isolates)	84 strong ps[b]	84 non-strong ps

Note: Ps: *Pseudomonas,* nps: non-*Pseudomonas*

[a]Paired sample *t*-test showed that this group has the highest statistical QQ activity. This result suggests that *Pseudomonas* isolates are generally stronger QBs compared with non-*Pseudomonas* genera.

[b]Paired sample *t*-test showed that this group has the highest statistical QQ activity, suggesting that the selection of strong bacteria was correct.

were inoculated with pathogenic bacteria. Control plates were inoculated solely with pathogens. The clear zones surrounding the spot cultures were considered as evidence of antibacterial activities. In addition, spot assays were performed against biosensor CV026 to ensure that there was no antibacterial activity against the biosensor as perturbation factors in QQ studies. For further verification, some isolates suspected to have this activity were tested again using a paper disk method. The disks were impregnated with each of the bacterial suspensions (thick bacterial suspension, more than 1×10^9 colony-forming units [CFU]/mL) inoculated in NA medium at three replicates, and the antibacterial activity was evaluated (Sammer et al., 2009).

2.7. Reduction of Pcc6-mediated potato rot

In the first step, we determined the biocontrol capacity of strong QBs and compared *Pseudomonas* and non-*Pseudomonas* biocontrol efficacy. Potato tubers were washed in tap water, followed by surface sterilisation using 70% ethanol. The sterilised tubers were sliced, and the strongest QB isolates and Pcc6 were grown in LB broth for 24 h. Subsequently, 100 µl of the *P. carotovorum* culture (the density was adjusted to 10^5 CFU/mL) was mixed with an equal volume of the QB isolates (10^5 CFU/mL). A 10-µl aliquot of the mixture was inoculated onto each slice, with four replications for each isolate. The incubated slices were stored in sealed plastic bags under moist conditions at 28°C (Dong et al., 2004). Slices inoculated with only Pcc and QBs were used as positive and negative controls (to ensure these bacteria cannot infect potato), respectively. Any reduction in the macerated region compared with the positive control was indicated as the QQ capacity of the respective strain. The percentages of reduction in potato soft rot were assessed against the control containing Pcc6 alone.

2.8. Verification of the biocontrol assay

The biocontrol efficacies of 84 frequent genera of strong QBs strains were compared with those of 84 frequent genera of non-strong QBs randomly selected among all isolates from both methods to confirm the direct relationship between QQ activity and biocontrol capacity.

2.9. Antipathogenic activity against other pathogens

The antipathogenic activities of strong QBs were evaluated against *E. amylovora* (Ea1 and Ea2), Pc8, and *F. solani*. The reductions in pathogenicity and antibacterial properties were

assessed according to a previous experiment performed for Pcc6 potato rot reduction and an antibacterial assay against Pcc6, respectively. However, the biocontrol assay against Ea1 and Ea2 was evaluated using pear fruits (the same volume of 10^5 CFU/mL QBs and pathogens), and the symptom zones on these fruits were compared among QBs (Figure 3D). In addition, a dual culture of QBs and fungal isolates was developed for antifungal activity assessment (Gupta, Dubey, Kang, & Maheshwari, 2001). Biocontrol comparisons among strong QBs against Ea1, Ea2, and Pc8 were performed as per Pcc6-induced potato rot reduction (Table 4).

2.10. Mechanism of QQ action in QBs

The QB cells were harvested from two-day-old cultures in Luria–Bertani (LB) medium at approximately 10^9 CFU/ml. After centrifugation, the cells were re-suspended in 100 ml of PBS and washed twice in PBS (supernatant stored for other experiments). The bacterial cells were disrupted using sonication, and the cell debris was removed through centrifugation at 16,000 *g* for 90 min. The resulting crude cell extracts (CCEs) were filtered (0.2 μM) and stored at 4°C (Uroz et al., 2005). The supernatants and CCEs of QBs were used in four tests: first, the supernatant was passed through filters (0.2 μm); second, the filtered supernatant was heated to 121°C for 15 min; third, the supernatant and CCE were treated with proteinase K; and fourth, to further investigate the mechanisms of QQ activity, the filtered QB supernatant from the first stage was passed through Microcon centrifugal filter tubes (Millipore corporation; 3 kDa-cut off). Subsequently, 3-oxo-C6-HSL was subjected to supernatants and CCEs of QBs (related to the four tests) in the presence of CV026 as described above in a biosensor assay to detect the presence or absence of remaining QQ activity. These tests were conducted for all strong QBs.

2.11. Verification of the frequencies of QQ mechanisms

In addition to the QQ mechanism assessments performed for strong QBs, these tests were randomly conducted for 21 rhizospheric and 21 phyllospheric frequent QB isolates for each method. These isolates were selected among all QBs, except strong QBs (totalling 84 randomly selected non-strong QB isolates), to determine the precise frequencies of mechanisms in the QB collection.

2.12. Statistical analysis

The experiments were performed in a completely randomised design, and for each bacterial isolate, 8 and 4 replications were, respectively, used for the biosensor and biocontrol assays. The data were analysed with SAS software (version 9.1) using analysis of variance (ANOVA). The numbers of QBs among three factors (method, niche, and plant) were calculated using Minitab (version 16.2) in a factorial experimental design. All pairwise comparisons related to QQ activity and biocontrol efficacy were conducted using SPSS (version 15.0). ANOVA and the nonparametric Mann–Whitney U-test were used to assess the normality and non-normality of the data, respectively. For ANOVA, Tukey's test (at $P < .05$) was used to identify significant differences between different treatments.

3. Results

3.1. Isolated QBs

Isolates with antagonistic activities against biosensors were excluded from further experiments. The MM method was more successful for the isolation of QB bacteria than the non-MM method. The different methods showed a significant difference in their QB-isolating strengths, such that the MM method isolated more QBs than the NM method in both niches and amongst the three plants (Figure 2). All QBs were detected amongst 6000 colonies of bacteria related to both methods (Figure 1).

3.2. Detection of the most abundant and strongest genus amongst the QBs

The greatest portions of QBs were isolated at 28, 30, and 25°C, respectively, and a few isolates were associated with other temperatures. Different temperatures did not show significant effects on the outcomes of the biosensor assays (data not shown). In addition, almost all randomly selected QBs associated with different temperatures grew normally in LB medium at 28°C. Therefore, the following experiments were adjusted to the described conditions. In the rhizosphere isolates, more than 70.9% of the isolated bacteria were Gram-negative, while 43.7% of the phyllospheric isolates were Gram-negative. Furthermore, these tests showed that more than 50% of the QB populations in six rhizospheric states (each row of Table 1 reflects one state) belonged to the *Pseudomonas* genus, which was more than the phyllospheric states. Nevertheless, a significant portion of QBs in this niche belonged to *Pseudomonas* (Table 1). The most *Pseudomonas* strains resulting from the NM method belonged to the pomegranate-rhizosphere state (Table 1; row 3 and column 3), while a significant proportion of QQ-*pseudomonades* with activity against O-C6 belonged to Fig-NM method-rhizosphere. In this state, *pseudomonades* constituted the highest percentage of QBs (64.4%; Table 1 and column 5) compared with other states. In addition, the highest percentage of the QB population with activity against O-C6 was isolated from fig-rhizospheres states related to NM and MM methods. Therefore, fig-rhizospheres contain the highest amounts of QBs and QQ-*Pseudomonas* among all states. A total of 127 QBs were indicated as strong QBs. More than 50% (84/127) of the strongest

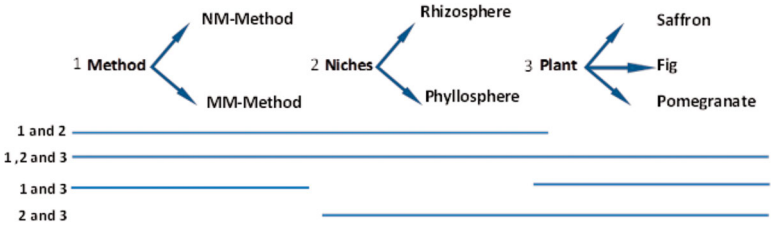

Figure 2. All single and interaction effects among the three factors are presented. The solid lines show interaction effects among the three factors (method, niche, and plant). The number of QBs in factor 1, the method, and all interaction effects with this factor were significantly different using Tukey's test (95.0% confidence). There were no significant differences between other factors. All single and interaction effects among the three factors in the average %QQ-*Pseudomonas* amongst all QBs with activity against OC6-HSL were significantly different.

QBs among all 12 states belonged to the *Pseudomonas* genus. Comparisons of the QQ activities between strong QBs showed that 84 strong QQ-*Pseudomonas* possessed significantly higher QQ activity levels than 43 strong non-*Pseudomonas* QBs (Table 2). The two strongest QQ-*Pseudomonas* strains were isolated from pomegranate samples (NM-R-P and MM-R-P), but QBs and QQ-*Pseudomonas* were most frequently isolated from fig samples (NM-R-F and MM-R-F). Generally, the populations of QQ-*Pseudomonas* were significantly different among various states, but a dominant percentage of QBs in many states belonged to these bacteria (Table 1).

3.3. Antibacterial and biocontrol activities against Pcc6

Antibacterial activity assessments showed that the quantity of *Pseudomonas* with QQ activity was much greater than that of *Pseudomonas* with antibiosis properties among all *pseudomonades* related to the NM method, which represented culturable bacterial populations. The QQ and antibiosis capacities were 29.75% and 8.3%, respectively (Table 1). Comparison of the biocontrol efficacies of 127 strong QBs (84 *pseudomonades* and 43 non-*Pseudomonas* strains) in the potato rot assay demonstrated that the QQ capacities of the QQ-*Pseudomonas* isolates were significantly stronger than those of the non-*pseudomonades* (Table 3). In addition, 13 of the 14 strongest biocontrol isolates belonged to the *Pseudomonas* genus. Only 4 strong QQ-*Pseudomonas* isolates (and 8 non-*Pseudomonas* isolates) did not show a biocontrol effect against Pcc6 (Table 4). Therefore, *Pseudomonas* strains are perceived to be the best and most abundant biocontrol agents among all QBs (Tables 1 and 4).

3.4. 3-oxo-C8-HSL-cleaving activity

The results of 3-oxo-C8-HSL-biosensor assays were similar to those of the 3-oxo-C6-HSL-biosensor assays for NM-R-F and MM-R-F states. The specific QQ activity was observed in only a few QQ-*Pseudomonas* isolates for two AHLs with different acyl chain lengths, indicating activity against 3-oxo-C6-HSL or 3-oxo-C8-HSL (Tables 1 and 4).

3.5. Antimicrobial and biocontrol activities against other pathogens

QQ-*pseudomonades* were stronger biocontrol agents (Table 3). Few QBs showed quenching activity against individual QS signals but did not show biocontrol effects on self-same signal-operating pathogens (Table 4). This contradiction might reflect the pathogenicity of

Table 3. Biocontrol assays for 127 strong QBs.

Bacterial pathogens	Number of isolates Ps–nps	Number of effective biocontrol agents Ps–nps	Number of non-effective biocontrol agents Ps–nps
Pcc6	84[a]–43	80–35	4–8
Pcc8	84[a]–43	73–23	11–20
Ea1	84[a]–43	73–31	11–12
Ea2	84[a]–43	68–24	16–19

[a]Paired sample *t*-test showed that this group has the highest statistical biocontrol efficacy, suggesting that *Pseudomonas* isolates are generally stronger QQ-based biocontrol agents compared with non-*Pseudomonas* isolates.

Table 4. Number of QBs in different tests (among 127 strong QBs).

Niches	Strong QB against Oc6 and Oc8	Strong QB with biocontrol ability or antifungal activity against Pcc6, Pcc8, Ea1, Ea2, fungus and all of them	*Pseudomonas* with both antibacterial and QQ properties against Pcc6[a]
	Ps-nps	Ps-nps	
NM-rhizosphere	10–2	10–2	1
	1–1	9–1	
		8–1	
		7–0	
		1–0	
		1–0	
NM-phyllosphere	4–5	4–4	0
	4–3	4–3	
		4–4	
		4–3	
		0–1	
		0–0	
MM-rhizosphere	42–15	39–12	1
	41–12	37–7	
		36–10	
		35–8	
		4–1	
		3–0	
MM-phyllosphere	28–21	27–16	0
	27–20	23–11	
		24–15	
		22–12	
		1–1	
		1–1	

Note: nps: non-*Pseudomonas* and ps: *Pseudomonas*, Oc6:oxo-c6-SHL, Oc8:oxo-c8-SHL, NM: NM method, MM: MM method.
[a]These QBs did not show antibiosis activity against a biosensor but did show activity against Pcc6.

QBs. For example, two non-*Pseudomonas* isolates with moderate pathogenicity were identified on pear (Figure 3 and Table 4). *Pseudomonas* isolates induced more biocontrol efficacy than non-*pseudomonades* on bacterial pathogens with different QS signals, as only 11 out of 84 strong *Pseudomonas* isolates (with high oxo-C6-HSL-degrading activity) did not show biocontrol activity against Pcc8. A total of 21 out of 43 strong non-*pseudomonades* isolates showed no biocontrol activity against Pcc8. Amongst the six versatile QBs with biocontrol capacity against all pathogens, five QBs were QQ-*pseudomonades* (Table 4).

3.6. Elucidation of the QQ mechanism

Stage 1, that is, filtration, was performed to identify QBs with extracellular QQ activity. Stages 2 and 3 were conducted to investigate the proteinaceous or non-proteinaceous QQ nature. Stage 4, using Microcon centrifugal filter tubes, was conducted to further confirm the non-proteinaceous (enzyme) nature of QQ isolates, which preserved this activity after heating and proteinase treatment in stages 2 and 3, respectively, suggesting a non-protein nature or thermo/proteinase-resistant protein. However, enzymes/proteins cannot easily pass through the filter. Although *pseudomonades* were identified as the strongest QQ-based biocontrol agents, a collection containing 84 non-strong QQ-*pseudomonades* was prepared to determine which QQ mechanism is more common in this

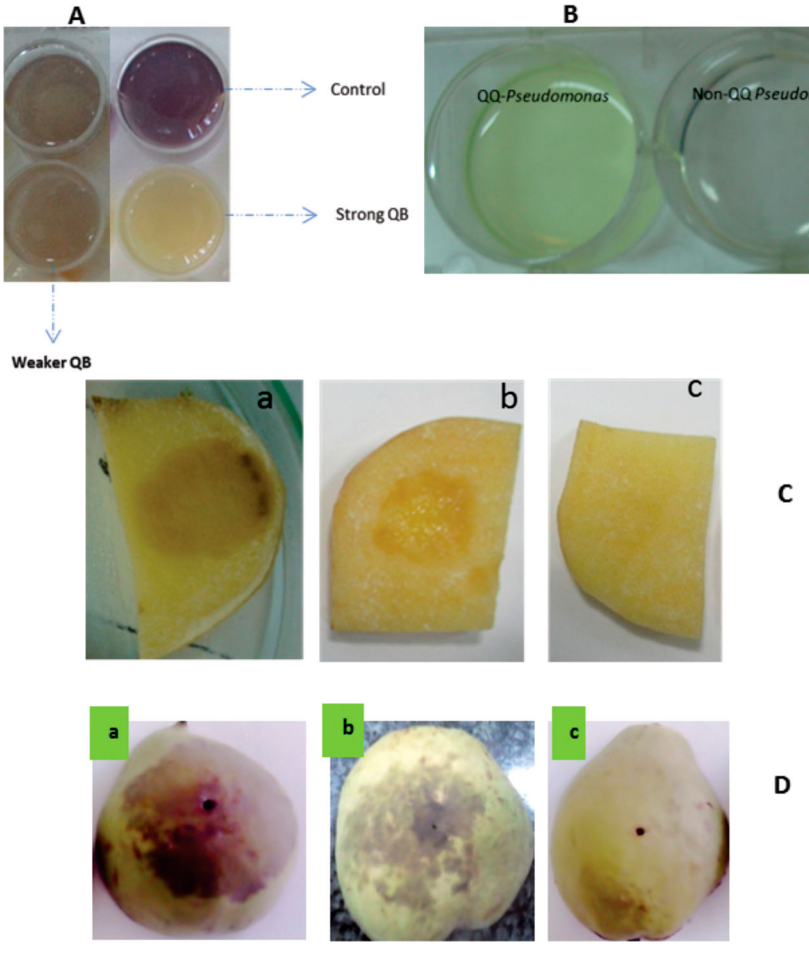

Figure 3. (A) AHL-inactivation assay: Purple- and non-purple-coloured wells in the cell cultures show negative control or non-QB and QB isolates, respectively. (B) Fluorescent test on CAA medium for the detection of fluorescent *Pseudomonas*. (C) Potato rot assay. a: Pcc alone as control, b and c: mix of different QB isolates and Pcc. (D) The biocontrol assay against Ea1 was evaluated on pear fruits. a: Ea1 alone as control, b and c: mix of different QB isolates and Ea1.

collection compared with 84 strong QQ-*pseudomonades* in terms of the frequency of each mechanism in both collections. One hundred sixteen out of 127 strong QBs completely lost their QQ capacity after stage 1. Furthermore, after stages 2 and 3 (heating and proteinase K treatment), the QQ activities were also lost (group 1). Therefore, these QBs likely had intracellular enzymatic QQ activities. Among all strong QQ bacteria, two QQ-*Pseudomonas* strains and three non-*pseudomonades* strains showed partly preserved QQ capacity after the four stages (group 3), and five QQ-*pseudomonades* and one non-*Pseudomonas* showed preserved capacities after stage 1 (group 2). Therefore, non-enzymatic small compound(s) and extracellular enzymatic QQ activities were involved in groups 3 and 2, respectively (Figure 4). Groups 2 and 3 of QBs in both collections of 84 strong QQ-*pseudomonades* (127 strong QBs) and 84 randomly selected non-strong QQ-*pseudomonades* were isolated using the NM method (Figure 4). Group 1 represented the most frequent mechanism among QQ-*pseudomonades* and other QBs, and many of

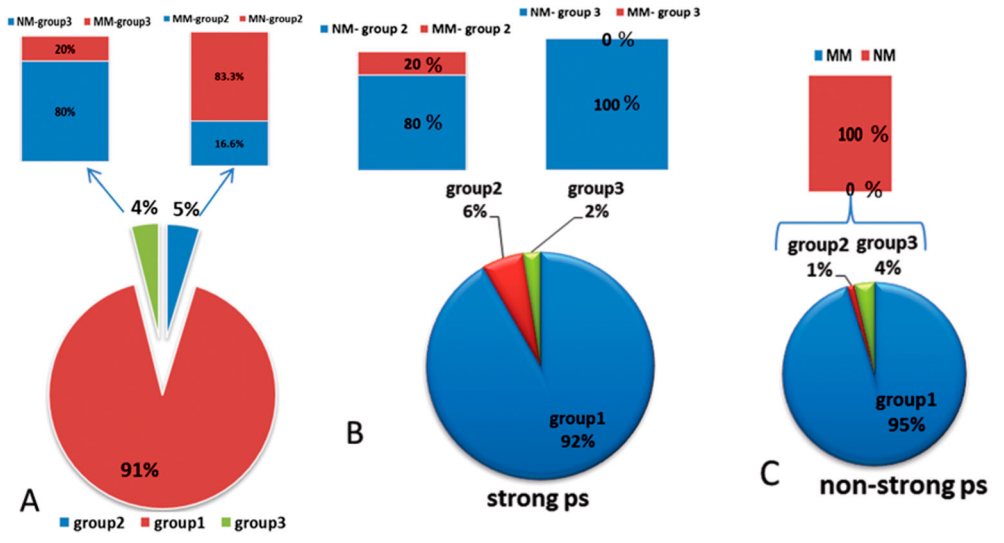

Figure 4. (A) Mechanism of QQ action in strong QBs. (B) Mechanism of QQ action in strong QQ-*Pseudomonas*. (C) Mechanism of QQ action in randomly selected non-strong QQ-*Pseudomonas*. Group 1: intracellular enzymatic QQ activity, group 2: extracellular enzymatic QQ activity, group 3: non-enzymatic QQ activity, NM: NM method and MM: MM method.

the highest and lowest biocontrol and QQ activities were associated with this group. Although two strong QQ-*pseudomonades* demonstrated non-enzymatic activities, these isolates were the QQ-*pseudomonades* with the lowest QQ activities and biocontrol efficacies among all 84 strong QQ-*pseudomonades*. In general, group 2 was primarily observed among the strong QQ-based biocontrol *Pseudomonas*, as 5 out of the 6 isolates at the top of the list of 127 strong QBs belonged to group 2 QQ-*Pseudomonas* (5 out of the 6 best biocontrol agents against all phytopathogenic bacteria). However, only one isolate from group 2 was observed in 84 non-strong QQ-*pseudomonades* with less biocontrol efficacy.

3.7. Verification tests

A comparison of the QQ activities of 84 strong QQ-*pseudomonades* with those of 84 randomly selected non-strong QQ-*pseudomonades* revealed that the former isolates had more QQ activity and also verified the selection of strong QBs (Table 2). The biosensor-based assays revealed that almost all 84 strong QQ-*pseudomonades* had more biocontrol efficacy compared with the 84 randomly selected QQ-*pseudomonades* (only one strain was excluded from this assay). Therefore, as expected, the QQ activity assay results were consistent with those for biocontrol efficacy. In 84 randomly selected non-strong QQ-*pseudomonades*, QBs isolates related to group 2 were less than those in 84 strong QQ-*pseudomonades* collection (Figure 4C). Therefore, this group was considered a strong QB.

4. Discussion

Gram-positive QBs, such as *Bacillus*, are important and abundant QQ bacteria (Lee et al., 2002; Park, Park, Ryu, Park, & Lee, 2008). Initially, we expected to identify Gram-positive

QBs as dominant quenchers, but the Gram assay and subsequent fluorescent tests demonstrated that Gram-negative populations were more frequent, and, at least in the rhizospheres of the studied plants in Iran, *Pseudomonas* are the dominant QBs. However, the proportion of QQ-*Pseudomonas* in the phyllospheres of these plants was less than that in the rhizospheres (less than 50%). For example, the pomegranate-phyllosphere had the lowest percentage of QQ-*Pseudomonas* (13.8%; Table 2), but many QBs from diverse genera have been reported (Dong et al., 2002; Molina et al., 2003; Park et al., 2006; Uroz et al., 2003; Xu, Byun, Dussen, & Duke, 2003). Therefore, even 13.8% (Table 1) of the total QBs constituted a considerable amount, as *Pseudomonas* was the most abundant QQ bacterial genus in the phyllospheres of these plants. Further studies should be conducted on the rhizospheres and phyllospheres of different plants in other areas to obtain more accurate estimations of the QQ-*Pseudomonas* percentage. Considering the application in plant pathology, it is possible to identify appropriate QB biocontrol agents using both methods. However, a simple method employing MM medium primarily isolates bacteria with intracellular enzymatic-based QQ, whereas the time-consuming NM method increases the likelihood of identifying QBs with varied mechanisms. These three mechanisms, identified in QQ-*Pseudomonas*, were previously reported in several QBs (Christiaen et al., 2011), and *Pseudomonas* isolates with extracellular QQ activities were also implicated as useful QBs in several studies in diverse fields, such as biofouling control (Cheong et al., 2013). However, there are few reports on the effects of QQ mechanism on biocontrol capacity. In the present study, QQ-*Pseudomonas* with extracellular enzymatic QQ activity was determined as the best QQ-based biocontrol agent. Therefore, the current study verified the importance of these isolates in plant pathology. Several studies have shown that some QBs exhibit QQ activity against different AHLs (LaSarre & Federle, 2013). Some *pseudomonades* or their acylase genes showed specificity and preference for AHL with ≥7 carbons in their acyl chains, but other strains, species, or their QQ genes had activities against AHL with ≥6 carbons (Bokhove, Jimenez, Quax, & Dijkstra, 2010; LaSarre & Federle, 2013; Shepherd & Lindow, 2009; Wahjudi et al., 2011). These findings confirmed that there are several QQ-*Pseudomonas* related to the latter group. The results of the present study indicated that QQ-*pseudomonades* interfered with QS depending on the AHL family molecules, 3-oxo-C6-HSL and 3-oxo-C8-HSL. However, this finding likely reflects the selective pressure of MM medium containing 3-oxo-C6-HSL, but the same results were observed using the NM method. Many *Pseudomonas* species with QQ capacity have been isolated from various ecological niches (Riaz, 2008). However, most of the studies in the field of the biocontrol capacity of this genus have focused on antibiosis, and various strains of the *Pseudomonas* genus with this capacity have been effective in the control of bacterial and non-bacterial plant pathogens (Cigna et al., 2015; Maldonado-González, Bakker, Prieto, & Mercado-Blanco, 2015; Rashid, Chowdhury, & Sultana, 2013; Thomashow & Bakker, 2015). The results of the present study confirmed that there are other abundant biocontrol capacities, namely QQ, in some rhizospheres and phyllospheres of *Pseudomonas* populations. With respect to the large population of QQ-*Pseudomonas* with antipathogenic capacity against non-bacterial plant pathogens with different mechanisms, QQ-*Pseudomonas* was the best bacterial genus for screening multifunctional biocontrol agents. Previous studies have attempted to procure multifunctional biocontrol agents with antipathogenic capacity against bacterial and non-bacterial pathogens using high-throughput and difficult

techniques. For example, an N-acyl-homoserine lactonase (aiiA) gene from *Bacillus thuringiensis* was introduced into *Burkholderia* sp. with antifungal and nitrogen fixation properties, and this engineered *Burkholderia* strain reduced the disease of rice seedling rot resulting from *Burkholderia glumae* infection through the inhibition of QS signal production (Cho et al., 2007). However, in the present study, we isolated multifunctional biocontrol agents using simple methods. Accordingly, we recommend that an initial screening to identify QQ activity among *Pseudomonas* species should be performed prior to other antipathogenic evaluations to identify more functional biological control agents with wider applications against various plant pathogens. The results of the present study showed that *Pseudomonas* is a biological control agent with diverse mechanisms in biocontrol, including intracellular enzyme (protein) QQ, extracellular enzyme (protein) QQ, non-enzyme (protein) QQ, and non-QQ antipathogenic activity. In addition, if QQ is considered as a biocontrol method, then *Pseudomonas* is the best agent for future studies.

5. Conclusion

(1) *Pseudomonas* was the most abundant and strongest QQ-based biocontrol agent in rhizospheric and phyllospheric niches, particularly rhizospheres.
(2) QBs with extracellular enzymatic QQ activities are among the best biocontrol agents.
(3) The NM method was used to effectively screen QBs with diverse mechanisms.
(4) *Pseudomonas* spp. with activities against AHLs with ≥6 carbons in their acyl chains are overabundant.
(5) There are multifunctional biocontrol agents among QQ-*Pseudomonas* collections.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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