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Research Article

Native *Pseudomonas protegens* strains reduce soft rot inducing ability in potato under storage conditions

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Abstract: Among rhizobacteria, plant-beneficial effects of *Pseudomonas* bacteria are known in agricultural ecosystems. Screening of fluorescent pseudomonad isolates obtained from potato rhizosphere led to selecting five bio-reagents capable of controlling soft rot disease caused by *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) (JX029052) in plate assay, on intact potato tubers and under storage challenges using preventative and curative applications. The biocontrol features related to the tested rhizospheric bacteria were also evaluated. According to phenotypic tests, the representative antagonistic strains belong to the fluorescent pseudomonads group and are placed in the *P. protegens* cluster based on 16S rRNA gene sequencing. Strains IrPs8 and IrPs18 are potent to produce siderophore, cyanide hydrogen, and protease, form strong biofilm and carry *phl*, *plt* and *prn* genes on their genomes corresponding to 2, 4-diacetylphloroglucinol, pyoluteorin and pyrrolnitrin, respectively. Treated tubers inoculated with IrPs8 and IrPs18 showed a reduction in the soft rot-inducing potency of Pcc by 63.4 and 65.5% in preventative and by 57.8 and 58.3% in curative tests, respectively, under storage conditions that confirmed the *in vivo* results. This study highlights the potential of rhizospheric *P. protegens* strains as beneficial bacteria that can be suggested as preservative coatings for potato tubers under storage conditions.

Keywords: Antibiotic biosynthesis genes, Biological control, *Pectobacterium carotovorum* subsp. *Carotovorum*, Tuber maceration

Introduction

Bacterial soft rot generally occurs in Pectobacteriaceae family, one of the primary diseases in agricultural ecosystems (Czajkowski *et al*., 2012). Once this challenging disease is initiated in favorable conditions, it can rapidly extend on storage organs within a short period, and the producers do not have many alternatives for control (Charkowski, 2015; 2018).

Although planting certified seed potato tubers is recommended as a primary preventative measurement to manage this global decay, application of several microbial agents consisting of antagonistic bacteria (Cronin *et al*., 1997; Kodakaramian and Zafari, 2010; Czajkowski *et al*., 2012; Raoul des Essarts *et al*., 2016; Gerayeli *et al*., 2018; Krzyzanowska *et al*., 2019), N-acyl homoserine lactone degrading bacteria (Krzyzanowska *et al*. 2012), phenolic

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compounds (Kang *et al*., 2018) and plant extracts and volatile essential oils (Hajian-Maleki *et al*., 2019) are presented as preventive and/or curative treatments.

Cronin and co-workers *(*1997) confirmed the inhibition ability of *Pseudomonas fluorescens* F113 against *Pectobacterium atrosepticum* by applying a rifampicin-resistant mutant and indicated that 2,4-diacetylphloroglucinol antibiotic has a key role in F113 *in vitro* antagonism. fluorescent pseudomonads isolated from the potato rhizosphere in Hamadan province, Iran, were found to inhibit the growth of Pcc. The tested antagonistic bacterial suspensions also reduced the rate of soft rot in the field (Khodakaramian and Zafari, 2010)

The biocontrol of *Dickeya* sp. was evaluated using an endophytic isolate, *Serratia plymuthica* A30, which reduced the pathogen population under greenhouse trials (Czajkowski *et al*., 2012). This aforementioned biocontrol isolate can survive at low temperatures and was presented as a successful agent for *D. solani* to prevent potato tuber maceration during storage (Hadizadeh *et al*., 2019). Moreover, *Pseudomonas* sp. isolate P482 (classified as *P. donghuensis*) obtained from tomato rhizosphere showed antibiosis potential against *Pectobacterium* and *Dickeya* species (Krzyzanowska *et al*., 2012) and was further analyzed for novel antimicrobial genes (Krzyzanowska *et al*., 2016). The *P. fluorescens* (PA3G8 and PA4C2) and *P. putida* (PA14H7) isolates also displayed suppressive effect on *D. dianthicola*, the causal agent of potato blackleg (Raoul des Essarts *et al*., 2016). A mixture of bacterial antagonists, including *Serratia*, *Enterobacter* and *Rahnella* species, was also tested as a tuber preserver coating against dominant soft rot species in Europe and suggested as a promising tool for biocontrol of soft rot Pectobacteriaceae (Krzyzanowska *et al*., 2019).

Among beneficial rhizobacteria, fluorescent pseudomonads are noticed as harmless commensals, displaying several intrinsic properties to control plant diseases (Weller, 2007). The ability to colonize the plant roots in sufficient numbers (Haas and Keel 2003), to compete efficiently for niche, nutrients (Lugtenberg *et al*. 2001) and iron (Hofte and Bakker, 2007) in comparison to phytopathogen in the rhizosphere, to produce an army of bioactive metabolites such as antibiotics, cyclic lipopeptides, surfactants, and volatile substances for pathogen suppression (Raaijmakers *et al*., 2010; Mishra and Arora, 2018), to secret lytic exoenzymes (Nagarajkumar *et al*., 2004), and to enhance plant growth and to induce plant systemic resistance (Haas and Defago, 2005; Bakker *et al*., 2007) are considered as the natural features of these group of bacteria which highlight them as effective biocontrol agents.

Pectobacterium carotovorum subsp. *carotovorum* (Pcc), an economically and broadly distributed macergen, is responsible for significant downgrading and losses of stored potatoes in Iran (Baghaee-Ravari *et al*., 2013). Although several investigations were performed for biocontrol of fungal disease using *Pseudomonas* isolates (Ahmadzadeh and Sharifi-Tehrani, 2009; Afsharmanesh *et al*., 2010; Keshavarz-Tohid *et al*., 2017), only one work so far has assessed the antibacterial activity of rhizospheric fluorescent pseudomonads as protection tools targeting soft rot disease (Khodakaramian and Zafari, 2010) in Iran.

In this context, the current study intended to (i) isolate and assess the inhibitory efficacy of fluorescent pseudomonads collected from main potato-producing areas towards prevailing storage soft rot agent in Iran, Pcc by plate assay and *in vivo* experiments on intact potato tubers (ii) characterize the antagonistic traits and antimicrobial coding genes of *Pseudomonas* isolates, (iii) examine their ability to control the development of soft rot disease under semi-storage conditions and (iv) identify the promising representative biocontrol strains using 16sRNA gene sequencing.

Materials and Methods

Isolation of antagonistic bacteria from potato rhizosphere

To isolate antagonistic bacteria from the potato rhizosphere, healthy plants from potato fields of five provinces in Iran (Khorasan-Razavi,

Hamadan, Esfahan, Kurdestan and Ardabil) were gathered. The rhizosphere samples (10 g) were mixed in 100 ml of isotonic saline solution (NaCl, 8 g/liter) on a rotary shaker for 30 min. After sedimentation, 0.1 mL of bacterial suspension was streaked on King's medium B agar (King *et al*. 1954) and incubated at 28 °C for 24 to 48 h. The purified fluorescent colonies were preserved in Lysogeny broth (LB) medium containing 25% glycerol at −80 °C.

Growth inhibition bioassay

Known local soft rot strain *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) (JX029052) (Baghaee-Ravari *et al*., 2013) was applied in all assays. *In vitro* antibiotic production of rhizospheric bacterial isolates (producers) against Pcc-JX029052 was done with minor modifications (Cronin *et al*., 1997). Ten µL of the bacterial cultures of each probable antagonistic isolate was spotted on the surface of solid LB in three replicates, incubated at 28 °C for 48 h. Chloroform (0.5 mL) was added to a filter paper and placed in the lid of an upside-down plate at room temperature for 1 h. After evaporation of chloroform vapor, Pcc suspension (10^8 CFU/mL) was sprayed on each plate. Zone of inhibition was measured 48 h later, and the experiment was repeated twice. The producer isolates were classified as 'weak' antagonists if the inhibition zone was 10 mm or less, 'medium' if the inhibition zone ranged from 14 to 28 mm, and 'strong' if the inhibition zone measured between 29 to 39 mm. *P. fluorescence* (CHA0) is a known biological agent used in all experiments for comparisons.

Screening for suppression of potato tuber maceration

The representative isolates belonging to medium and strong antagonistic groups were tested for reducing tuber maceration development. For *in vivo* trials, certified seed potato tuber variety Agria (from Seed and Plant Certification and Registration Institute, Karaj, Iran) was surface-sterilized with 5% sodium hypochlorite for 15 min, washed 3 times with sterile distilled water to eliminate saprophyte pathogen present at their surface and residual sodium hypochlorite and finally air-dried. Subsequently, 20 µL of overnight bacterial suspensions of Pcc/antagonist (1:1 ratio) that had been cultivated in TY medium (Bacto tryptone, 5 g/L; yeast extract, 3 g/L; agar, 15 g/L) were stab inoculated into the potato tubers at a constant depth of 1.5 cm (Raoul des Essarts *et al*., 2016; Marquez-Villavincencio *et al*., 2011). Tubers inoculated with Pcc suspensions were considered solely positive controls. Treated tubers were stored at 25 °C in humidified plastic trays for 48 h. The rotted tissue was removed by scooping it out and weighted. Each treatment consisted of five replicates, and the experiment was repeated three times. The degree of rotting tissue was calculated following the formula of Kang *et al*. (2018): Decay (%) = (DW/IW) × 100; the formula IW means the initial weight of the tubers and DW related to the weight of decayed tissue after 48 h. The rotted tissue was removed by scraping; its mass was determined by weighing each Petri dish before and after removing the rots.

Application of antagonistic isolates against Pcc under semi-storage conditions

This assay consisted of preventative and curative treatments. All determinations were done at least twice, and eight tubers were used for each treatment. For this purpose, almost equal-sized potato tubers of various Agria were sterilized, as described earlier. Disinfected tubers were wounded artificially on each side along the longitudinal axis by creating a well using a thin sterile borer, as an infection site. Pathogen and candidate antagonists were grown in LB agar medium at 28 °C with shaking overnight. The bacterial cells were harvested and suspended in sterile water to obtain 10^7 and 10^8 CFU/mL^{-1} turbidity for Pcc and antagonist isolates, respectively. In the preventative trial, tubers were immersed in the antagonist suspension for 20 min and left at room temperature until dried. Pathogen inoculation was made by adding 15 µL of bacterial suspensions to each wound. Control potato tubers soaked in sterile water and then subjected to pathogen challenge. To investigate

the curative assay, 12 h after potato infection, the tubers were dipped in the antagonist suspension, as mentioned above. As positive controls, tubers infected by Pcc, were suspended in sterile water for 20 min. Moreover, uninoculated tubers soaked in sterile water were considered negative controls. All tubers were placed in plastic trays and maintained at a relatively high humidity for seven days. At the end of the experiment, the treated tubers were cut along the longitudinal axis and across the inoculation site to measure parameters including decay diameter (D, mm) and depth (d, mm). Following the formula of Lapwood *et al*. (1984), the penetration of the Pcc into the tubers is calculated;

$$P\ (mm) = [(D/2) + (d-6)]/2$$

In addition, the amount of RDI (Reduction in Disease Incidence) was obtained based on the formula presented by Sameza *et al*. (2016); %

$$RDI = (D_{Pcc} - D_{Pcc+antagonist}) / D_{Pcc} \times 100$$

Biochemical screening for siderophore, hydrogen cyanide production and enzymatic activity

All assays were performed twice, and the average values of three replicates were considered the final result. Siderophore production was semi-quantified using Chrome Azurol S (CAS) blue agar medium (Schwyn and Neilands, 1987). Following incubation at 28 °C for 3 days, the development of orange halos around the colonies was measured.

To detect cyanide, tested isolates were streaked on KB-agar medium amended with 4.4 g/l glycine. A sterilized filter paper impregnated with 0.5% picric acid and 2% sodium carbonate solutions was kept in the upper lid of each petri dish. Following sealing the plates with parafilm, they were incubated at 28 °C for 2 days (Bakker and Schippers, 1986). The development of a reddish-brown color indicates microbial production of cyanide. An uninoculated KB medium was used as a negative control.

The enzymatic activities of antagonists were ascertained by semi-quantitative measurement of probable haloes (in the presence or absence of an indicator) in Petri plate assays. The protease production was checked on a medium with 1% skim milk (Simbert and Krieg, 1994). Following 24-48 h, a clear halo around the colony was indicated as a positive reaction. Cellulase activity was screened as described by Hankin and Anagnostakis (1977) using 1% carboxymethyl cellulose as substrate and 1% Congo red solution as an indicator. Chitinase secretion was tested on a semi-minimal medium with 0.2% colloidal chitin, followed by applying 1% Congo red and then washing with 1 M NaCl (Murthy and Bleakley, 2012).

Microtiter-plate test for biofilm formation

The adhesion potential of studied antagonists to produce biofilm was evaluated by 96-well polystyrene plates as described by O'Toole and Kolter (1998). Each well was filled with 200 µL of bacterial suspension in LB medium and incubated at 28 ºC for 36 h without agitation. Following incubation, the wells were washed with deionized water three times and vigorously shaken to remove all unattached bacteria. Adhering cells were stained with 0.1% w/v crystal violet and then rinsed under tap water. Subsequently, 200 µL of 99% methanol was added to solubilize the crystal violet. The absorbance of the solubilized dye was measured at 570 nm using a plate reader. This trial considered two independent assays with three replicates for each isolate.

PCR screening of antibiotic genes in candidate antagonists

The presence of four antibiotic biosynthesis genes in fluorescent pseudomonads, including *phl*D (2,4 -diacetylphloroglucinol) and *phz*CD (phenazine) (Raaijmakers *et al*., 1997), *plt* (pyoluteorin), *prn* (pyrrolnitrin) (Mavrodi *et al*., 2001) was evaluated according to literature. Briefly, PCR amplification was carried out with an Applied Biosystems 2720 thermocycler in a 25-µL reaction mixture containing 1 × Taq DNA polymerase buffer, 200 µM of dNTPs, 20 pmol of each primer, 1.5 mM $MgCl_2$, and 2.0 U of *Taq* polymerase (Cinnagen, Tehran) and 50 ng DNA. The PCR cycling programs for antibiotic genes consisted of an initial denaturation at 95 °C for 2

min, followed by 30 cycles at 95 °C for 1 min, annealing at 64 °C (*phl*D),56 °C (*phz*), 68 °C (*prn*), 67 °C (*plt*), for 1 min, and extension at 72 °C for 1 min. Standard reference *P. fluorescens* CHA0 was used as a positive control in detecting *phl*D, *plt* and *prn* genes.

Key biochemical tests

The selected *Pseudomonas* isolates were characterized using LOPAT assays, including levan formation, oxidase production, potato soft rot potential, arginine dehydrolase secretion and hypersensitivity of tobacco leaves following Schaad guidelines (Schaad *et al*., 2001). Moreover, growth at 4 and 41 ºC, nitrate reduction, gelatin hydrolysis and utilization of carbon sources such as L-arabinose, L-tartrate, sorbitol and trehalose were investigated according to Bossis *et al*. (2000). All tests were done two times with four replicates.

Molecular identification of candidate antagonistic pseudomonads

Bacterial isolates were grown overnight with shaking (150 rpm) in 20 mL of liquid LB medium for genomic DNA extraction. DNA was extracted using DNA extraction kit (Yekta-Tajhiz, Iran), following the manufacturer's instructions. DNA was quantified spectrophotometrically and adjusted to 30 ng $\mu L^{-1.}$ Amplifications of the 16S rRNA gene were employed using universal primers fD1/rD1 (Weisburg *et al*., 1991) by standard PCR. The amplified fragments were purified and sequenced in both directions using an automatic sequencer 3730X (Macrogen, Korea). A set of comparable sequences for 16S rDNA from the NCBI GenBank database was aligned with our obtained sequences using ClustalX 1.81 (Thompson *et al.*, 1997). A neighbor-joining phylogenetic tree was constructed with bootstrap analysis based on 1000 replicates in MEGA 7 software (Kumar *et al.*, 2018).

Statistical analysis

The SPSS statistical package (version 22.0, SPSS Inc., Chicago, IL, USA) was used for data processing. Results were expressed as mean ± standard deviations by one-way analysis of variance (ANOVA), and a comparison of means was performed using Duncan's multiple range test at 5% significance level.

Results

Growth inhibition assay of Pcc by fluorescent pseudomonads

Among 152 fluorescent pseudomonads obtained from potato rhizosphere in the current study, 18 strains exhibited inhibition halo towards tested Pcc (JX029052) in a primary agar plate assay (Figs. 1 and 2). Four strains were grouped as weak antagonists (inhibition diameter ≤ 10 mm) and were not retained for further analyses. The diameter of the inhibition zone between 14 to 28 mm (designed as medium antagonists) was measured in eight strains (44.4%). In addition, the diameter of growth inhibition zones in CHA0 and six strong antibiotic producers (IrPs2, IrPs5, IrPs9, IrPs10, IrPs17, and IrPs18) was recorded in the range of 29-39 mm. The last two groups were chosen for further studies.

Potato tissue maceration ability of Pcc in the presence of antagonistic fluorescent pseudomonads

The activity of 14 antagonists was evaluated for reduction of maceration development on individual tubers by Pcc (JX029052) *in vivo*. No fluorescent pseudomonads could induce soft rot symptoms. Although the protective effect was shown in the case of all antagonistic bacteria (Fig. 3), five isolates besides CHA0 could attenuate the infection incidence of Pcc strain in the range of 1.69 to 6.05 times compared with positive control, as depicted in Fig. 3.

Effect of fluorescent pseudomonads on the reduction of soft rot incidence during storage

The impact of 14 antagonistic isolates on soft rot disease during storage under preventive and curative assays is summarized in Table 1. After applying all fluorescent pseudomonads isolates, soft rot development decreased in both assays

(compared to Pcc -inoculated tubers). The preventive experiment reduced disease incidence and pathogen penetration in the tuber wounds greater than the curative test. Although the Pcc-coated tubers had high rot penetration (8.25 mm), no decay was observed in the individual application of antagonistic isolate. The lowest average penetration was shown in potato tubers coated with IrPs9 and IrPs18 before JX029052 (2.13 and 2.25 mm) inoculation (Fig. 4). The curative effect of IrPs9 and IrPs18 coating on potato tubers that had been inoculated with Pcc strain was also recorded as satisfactory (2.6 mm in combination with JX029052).

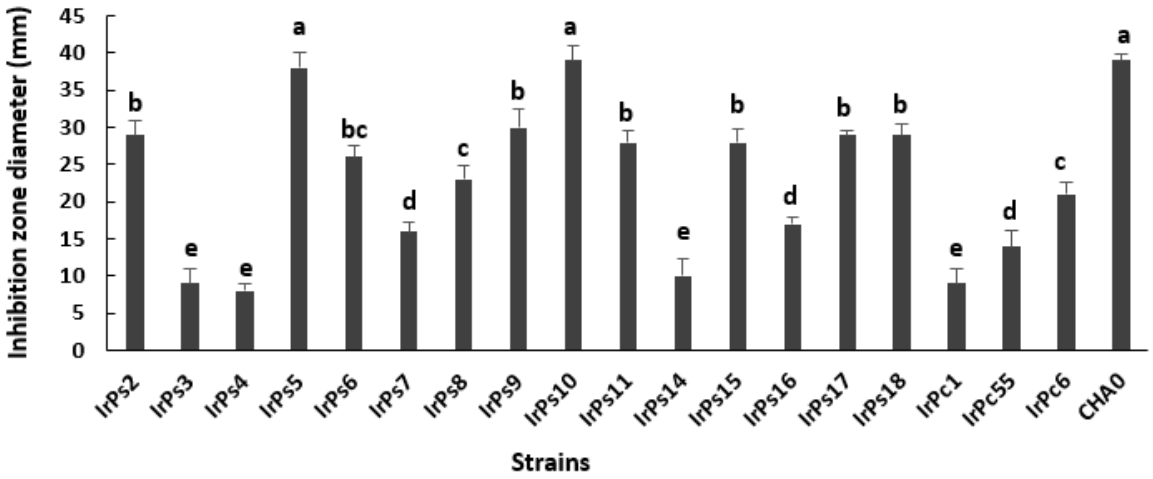

Figure 1 Antibacterial activity of fluorescent *Pseudomonas* isolates obtained from potato rhizosphere against *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc-JX029052) in the plate assay after 48 h. Means with different letters indicate significant difference (Duncan's multiple rage test, $P \leq 0.05$).

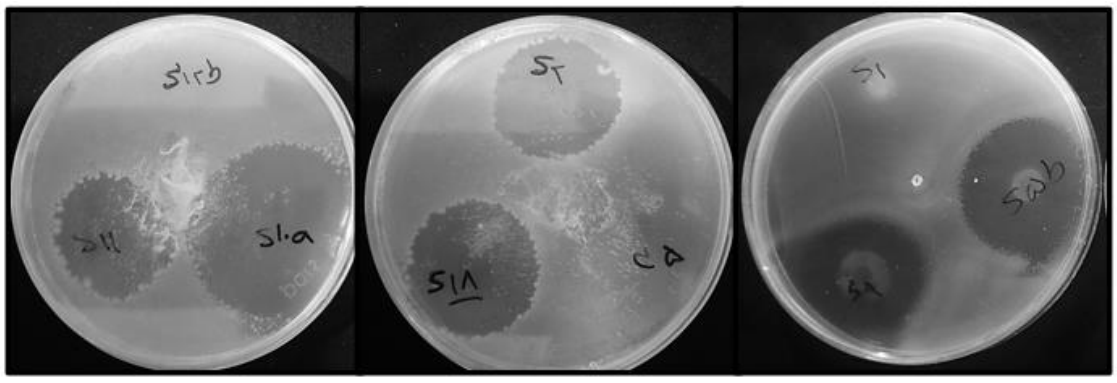

Figure 2 *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc-JX029052) growth inhibition at the presence of candidate fluorescent *Pseudomonas* isolates obtained from potato rhizosphere in plate assay after 48 h.

The reduction in disease incidence (RDI) ranged from 28.5% to 65.5% when fluorescent *Pseudomonas* isolates were used preventively and from 24.8% to 58.3% in curative treatments. Overall, both experiments showed that the highest RDI values for the *Pcc* indicator strain were associated with isolates IrPs18, IrPs8, IrPs9, IrPs10, and IrPs5, compared to other native fluorescent *Pseudomonas* isolates. Additionally, the reference strain CHA0 demonstrated a satisfactory ability to protect and treat potato tubers from *Pcc* infection, as shown in Table 1.

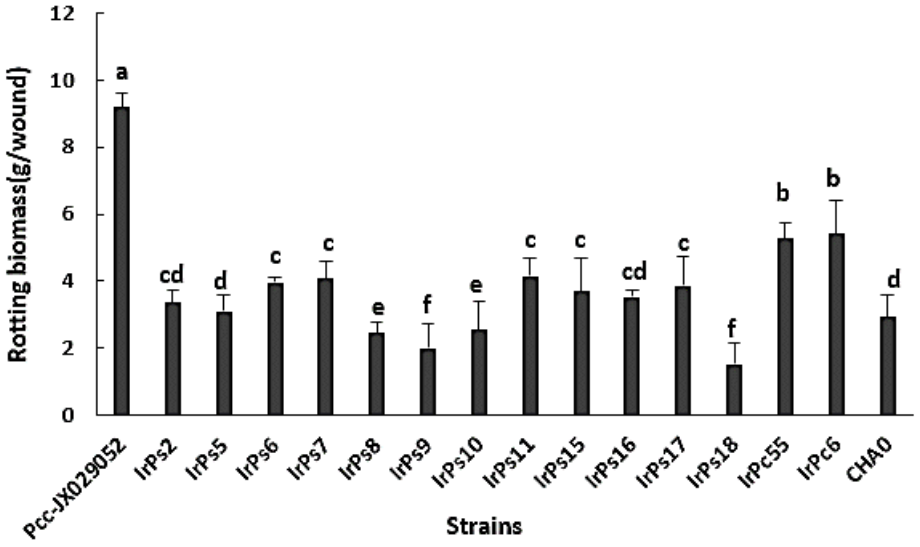

Figure 3 The efficacy of fluorescent pseudomonads isolates obtained from potato rhizosphere on Pcc-JX029052 rotting ability after 48 h under *in vivo* conditions. The data were obtained from five repetitions and values with the identical characters are not significantly different (Duncan's multiple rage test, $P \leq 0.05$).

Table 1 The potential of fluorescent *Pseudomonas* isolates obtained from potato rhizosphere on soft rot development in wounded potato tubers inoculated by Pcc-JX029052 under storage conditions. The tubers were soaked in the antagonist's suspension before or after Pcc-JX029052 inoculation.

Antagonists	Preventative assay		Curative assay	
	RDI (%)[1]	P (mm)[2]	RDI (%)	P (mm)
IrPs2	43.5 ± 0.87 d	4.2 ± 0.08 c	38.6 ± 1.45 c	4.8 ± 0.58 d
IrPs5	59.09 ± 0.67 b	2.8 ± 0.12 d	53.8 ± 0.98 b	3.1 ± 0.17
IrPs6	41.8 ± 0.26 d	4.5 ± 0.32 c	36.2 ± 1.15 c	5.3 ± 0.64 c
IrPs7	42.7 ± 1.12 d	3.8 ± 0.10 cd	36.9 ± 0.97 c	4.5 ± 0.65 d
IrPs8	63.4 ± 0.84 a	2.75 ± 0.42 d	57.8 ± 1.39 a	3.2 ± 0.87 e
IrPs9	63.7 ± 1.08 a	2.13 ± 0.06 e	58.3 ± 0.84 a	2.6 ± 0.93 f
IrPs10	55.2 ± 0.46 c	2.58 ± 0.08 d	50.7 ± 0.86 b	2.9 ± 0.13 f
IrPs11	36.2 ± 0.91 e	5.3 ± 0.44 b	31.5 ± 0.46 e	6.2 ± 0.26 b
IrPs15	39.3 ± 0.51 de	4.6 ± 0.34 c	34.6 ± 0.67 d	5.7 ± 0.52 c
IrPs16	38.4 ± 1.36 de	3.8 ± 0.12 cd	29.7 ± 0.96 e	4.6 ± 0.49 d
IrPs17	40.1 ± 1.67 d	4.2 ± 0.46 c	32.5 ± 1.85 e	4.8 ± 0.75 d
IrPs18	65.5 ± 1.10 a	2.25 ± 0.08 e	58.3 ± 1.73 a	2.6 ± 0.08 f
IrPc5	30.7 ± 0.45 f	5.2 ± 0.78 b	26.6 ± 1.64 f	6 ± 0.77 b
IrPc6	28.5 ± 0.77 f	5.6 ± 0.27 b	24.8 ± 0.96 f	6.3 ± 0.18 b
CHA0	61.45 ± 0.51 b	2.89 ± 0.36 d	57.8 ± 0.78 a	3.1 ± 0.34 e
Pcc	-	8.25 ± 0.57 a	-	8.25 ± 0.76 a

[1] Reduction in disease incidence (RDI) and [2]penetration depth (P) were measured after one week and compared with the controls in preventative and curative treatments. Average values and standard deviations related to eight measurements. Different lowercase letters in each column indicate significant difference (Duncan's multiple rage test, $P \leq 0.05$).

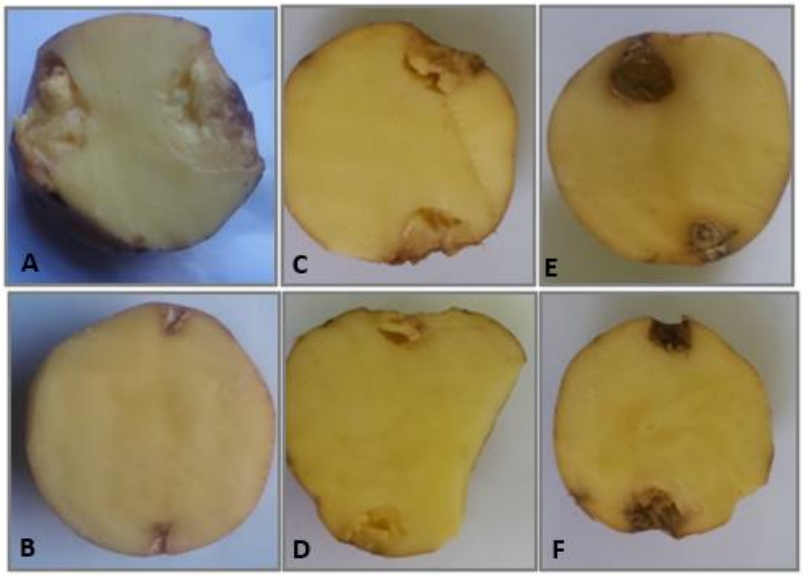

Figure 4 The rhizospheric fluorescent *Pseudomonas* bacteria could decrease disease incidence in potato tubers inoculated by Pcc-JX029052 under storage conditions. A: Pcc-JX029052; B: Distilled water; C: IrPs9 + Pcc-JX029052 (curative treatment); D: IRPs9 + Pcc-JX029052 (preventative treatment); E: IrPs18 + Pcc-JX029052 (curative treatment); F: IRPs18 + Pcc-JX029052 (preventative treatment).

The biocontrol properties of fluorescent *Pseudomonas* isolates

As mentioned in Table 1, the 14 bacterial isolates besides *P. protegens* CHA0 could produce siderophore. Thirteen out of 14 tested isolates showed high production of hydrogen cyanide (filter paper color change from yellow to reddish-brown). Although protease activity was recorded in all isolates as a degradation halo in the range of 15 to ≥ 30 mm in skim milk medium, no cellulase activity was seen for these isolates. Chitinase activity was detected in 42.86% of the tested isolates.

The biofilm formation rate of fourteen bacterial isolates beside *P. protegens* CHA0 was obtained by measuring the crystal violet dye intensity adhered to microtiter plate wells (Fig. 5). The highest rate of biofilm formation was determined for isolates IrPs18, IrPs15, IrPs8 and IrPs5.

Five candidate fluorescent *Pseudomonas* isolates were selected based on the amount of inhibition on plate assay and reduction in the rate of tuber decay under *in vivo* and storage conditions. They were chosen to evaluate the presence of known antibiotic biosynthesis genes (Table 2). *P. protegens* CHA0 (*phl*$^+$, *plt*$^+$, *prn*$^+$) was used as a control. Although the gene corresponding to 2,4-diacetylphloroglucinol was detected in all five candidate antagonists, *plt* gene was amplified in IrPs8 and IrPs18. The expected 786 bp PCR amplified fragment related to pyrrolnitrin was seen in IrPs8, IrPs9 and IrPs18. No PCR product was detected for phenazine in tested isolates.

Biochemical and physiological identification

According to phenotypic traits (Table 3), five candidate biocontrol isolates had oxidase activity and produced arginine dihydrolase. No pectinase activity nor hypersensitive response induction on tobacco leaves was seen, and the levan production was negative except for IrPs8 and IrPs18. None of them showed ice nucleation activity. They all grew at 4 °C but did not develop at 41 °C, hydrolyzed gelatin and utilized trehalose and L-tartrate. The utilization of nitrate reduction and carbon sources (sorbitol and D-xylose) varied among these isolates. IrPs5 and IrPs9 (positive nitrate reduction) were identified as *P. fluorescence* bv. III. IrPs10 (negative nitrate reduction and levan) and IrPs18 (negative nitrate reduction and levan positive) were characterized as *P. fluorescence* bv. V and I, respectively. The

last biocontrol isolate, IrPs8 belonged to *P. fluorescence* bv. IV is based on its positive reaction in levan production, nitrate reduction, and utilization of L-tartrate and sorbitol.

16s rRNA gene sequencing of the candidate *Pseudomonas* strains

According to the 16S rRNA gene sequences related to our native isolates and other sequences retrieved from GenBank, the studied *Pseudomonas* isolates were clustered with the relevant sequences of *P. protegens* (Fig. 6). Strains associated with other fluorescent *Pseudomonas*, including *putida*, *chlororaphis* and *syringae* were grouped separately.

Discussion

Dickeya and *Pectobacterium*, as soft rot plant pathogens, are listed among the ten top economically important bacterial plant pathogens (Mansfield *et al*., 2012) and are responsible for diseases in various important crops worldwide (Czajkowski *et al*., 2011). Seed potatoes and, to some extent, soilborne inoculum were previously considered the most important source of disease spread (Van der Wolf *et al*., 2021; Nykyri *et al*., 2014). Moreover, the entrance of the pathogen via airborne inoculum carried by rain, aerosols, insects, machines, footwear, animals, or laborers is characterized as more possible infectious ways (Kastelein *et al*., 2020). Although five macergens, including *D. solani*, *P. atrosepticum*, *P. brasiliense*, *P. carotovorum* and *P. parmentieri* (formerly *P. wasabiae*) causes severe losses in many vegetables in fields, storages and transit worldwide (Degefu, 2024), *P. carotovorum* subsp. *carotovorum* is a prevalent bacterial potato pathogen in storage in Iran (Baghaee-Ravari *et al*., 2013).

In sustainable agriculture, global concerns are increased due to synthetic chemical residues in the environment and foods (Palmieri *et al*., 2022). So, applying microbial inoculants as promising tools is promoting to ensure plant health and the quality and safety of vegetal products (Pirttila *et al*., 2021). Among rhizospheric bacteria, *Pseudomonas* spp. display many traits that contribute to their biocontrol potential and strengthen plant defense (Panpatte *et al*., 2016). The *P. fluorescens* lineage comprises five phylogenetic groups and eight subgroups (Lalucat *et al*., 2020). Within the *P. fluorescens* group, *P. chlororaphis*, *P. protegens* and *P. corrugata* subgroups, are rich in biocontrol agents and properties (Vacheron *et al*., 2016).

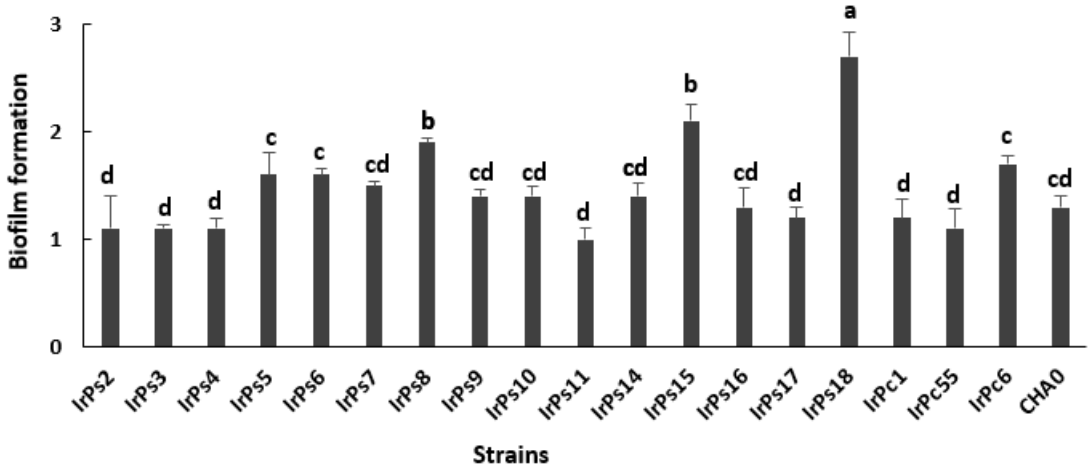

Figure 5 Biofilm formation of fluorescent *Pseudomonas* isolates obtained from potato rhizosphere against potato soft rot agent *(Pectobacterium carotovorum* subsp. *carotovorum*- JX029052) using microtiter plate assay. Quantification was done based on measuring optical density at 570 nm. Bars show standard errors of the means of three replicates. Means with different letters indicate significant difference (Duncan's multiple rage test, $P \leq 0.05$).

Table 2 Some antagonistic properties of fluorescent pseudomonads obtained from potato rhizosphere against potato soft rot agent *Pectobacterium carotovorum* subsp. *carotovorum*- JX029052.

Strains [1]	Siderophore [2]	HCN [3]	Enzymatic activity [4]			Antibiotic biosynthesis genes [5]			
			Protease	Cellulase	Chitinase	*phl*	*phz*	*plt*	*prn*
IrPs2	3.5 ± 1.34 b	+++	+	-	-				
IrPs5	2.6 ± 0.88 c	+++	+	-	+	+	-	-	-
IrPs6	2.8 ± 1.12 c	+++	+	-	+				
IrPs7	3.2 ± 0.56 bc	++	+	-	+				
IrPs8	3.2 ± 0.45 bc	+++	+	-	-	+	-	+	+
IrPs9	2.9 ± 0.76	+++	+	-	-	+	-	-	+
IrPs10	3.4 ± 0.13 b	+++	++	-	+	+	-	-	-
IrPs11	3 ± 0.53 bc	+++	+	-	-				
IrPs15	2.8 ± 1.35 c	+++	++	-	-				
IrPs16	2.8 ± 0.96 c	+++	+	-	-				
IrPs17	2.9 ± 1.57 c	+++	+	-	-				
IrPs18	3.2 ± 1.78 bc	+++	+	-	-	+	-	+	+
IrPc5	3.6 ± 1.48 ab	+	++	-	+				
IrPc6	3.9 ± 0.77 a	+++	+	-	+				
CHA0	2.9 ± 0.83 c	+++	+	-	-	+	-	+	+

[1] Candidate antagonists showed anti-Pcc ability were obtained from potato rhizosphere.

[2] The orange halo around the colonies on Chrome Azurol S (CAS) blue agar medium was considered as a positive reaction and measured the diameter of halo in cm.

[3] A change in the color of the filter paper from yellow to light brown, brown, and reddish-brown was recorded as weak (+), moderate (++), and strong (+++) reaction respectively.

[4] Protease, cellulase and Chitinase were determined as a clear halo on a related media supplemented with skim milk, carboxymethylcellulose and chitin, respectively. (-) no activity; (+) halo range between 15 to 20 mm; (++) halo rage between 21 to 30 mm; (+++) halo range more than 30 mm.

[5] The distribution of antibiotic biosynthesis genes including *Phl* (2,4-diacetylphloroglucinol); *phz* (phenazine); *plt* (pyoluteorin) and *prn* (pyrrolnitrin) in tested Pseudomonas isolates was checked by PCR.

Table 3 Biochemical and physiological traits of candidate fluorescent *Pseudomonas* isolates obtained from potato rhizosphere show antagonism against potato soft rot agent *Pectobacterium carotovorum* subsp. *carotovorum*- JX029052.

Characteristics	**IrPs5**	**IrPs8**	**IrPs9**	**IrPs10**	**IrPs18**
Fluorescent pigment on KB medium	+	+	+	+	+
Ice nucleation activity	-	-	-	-	-
Levane production	-	+	-	-	+
Oxidase activity	+	+	+	+	+
Pectolytic activity	-	-	-	-	-
Arginine dihydrolase	+	+	+	+	+
Hypersensitive response on Tobacco	-	-	-	-	-
Nitrate reduction	+	+	+	-	-
Gelatine hydrolysis	+	+	+	+	+
Growth at 4 ºC	+	+	+	+	+
Growth at 41 ºC	-	-	-	-	-
Utilization of carbon sources:					
Sorbitol	-	+	+	-	+
Trehalose	+	+	+	+	+
D-Xylose	-	+	+	+	+
L-Tartrate	+	+	+	+	+

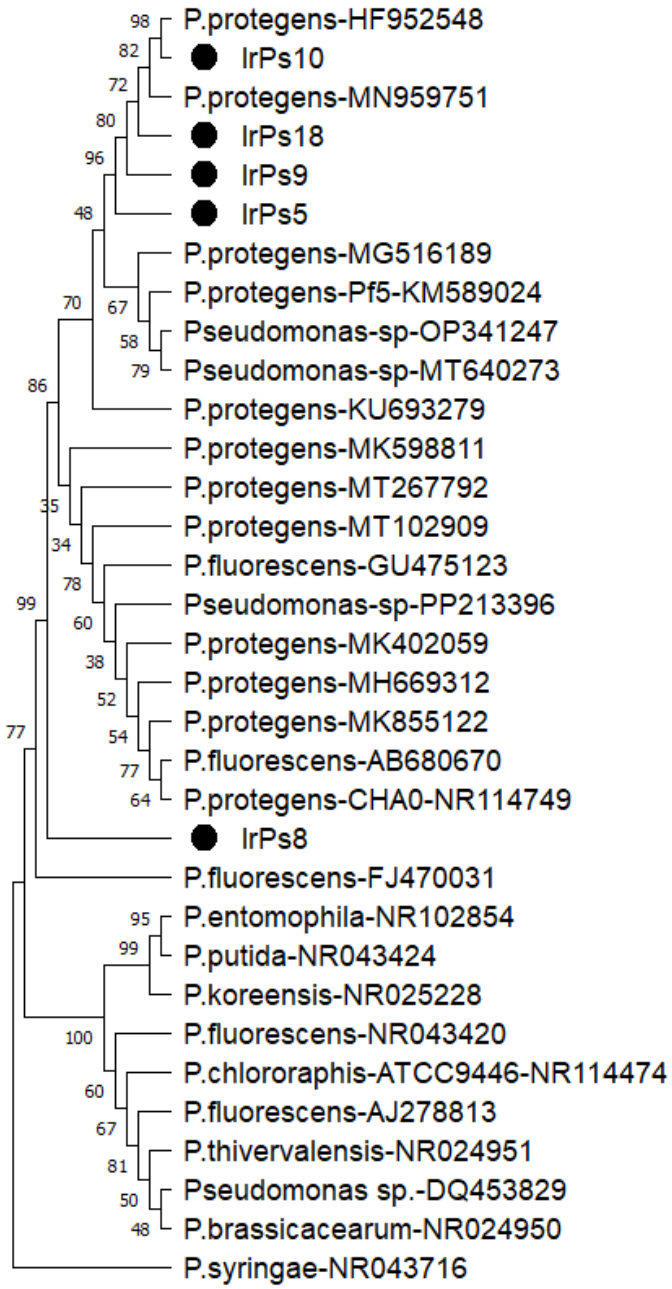

Figure 6 Phylogenetic analysis based on the partial 16S rRNA gene sequences of fluorescent *Pseudomonas* strains obtained from potato rhizosphere shows antagonism against potato soft rot agent *(Pectobacterium carotovorum* subsp. *carotovorum*- JX029052) (●) and other related strains retrieved from GenBank using neighbor-joining method. Numbers at the branches denote the bootstrap percentages for 1000 replicates.

Herein, in plate assays, five out of 18 fluorescent *Pseudomonas* show considerable inhibitory ability against Pcc (JX029052). Pre-treated tubers with antagonists mentioned above exhibited the highest potential to reduce rot development *in vivo*. All five selected isolates decrease the soft rot incidence in the range of 63.4-65.5% in preventative and 57.8-58.3% in

curative trials. These isolates also reduced the pathogen penetration depth (2.13-2.75 mm in preventative and 2.6-3.2 mm in curative assays) compared with the positive control (8.25 mm) under storage conditions. Based on *in vivo* data, coating potato tubers with antagonists before storage attenuated the rate of maceration by Pcc more than the curative test. Applying cultural filtrate of *P. fluorescens* reduced soft rot symptoms on treated potato slices when applied 2 h before or at the same time as pathogen inoculation (Abd-El-Khair *et al*., 2021).

The phenotypic properties of studied isolates were matched with the literature (Bossis *et al*., 2000; Schaad *et al*., 2001). The obtained 16s rRNA gene sequencing data indicated that the representative antagonistic strains belong to the *P. fluorescens* group and are placed in the *P. protegens* cluster. Although the production of siderophore, HCN, and protease, forming biofilm and detecting *phl* gene was positive in all five strains, the chitinolytic ability was recorded only in IrPs5 and IrPs10. Other antibiotic biosynthesis genes of *plt* and *prn* were screened in IrPs8 and IrPs18 strains.

In the whole potato tubers method, IrPs18 and IrPs8 highly protected the stored potato tuber under artificial infection conditions compared with other tested antagonists and positive controls. *P. protegens*-IrPs18 and IrPs8 strains are potent in producing siderophore, cyanide hydrogen, protease, forming biofilm and carrying *phl*, *plt* and *prn* on their genomes. Production of microbial siderophore helps sequester Fe^{3+} for host plants (Ma *et al*., 2011) and pathogen proliferation (Ahmad *et al*., 2008). Hydrogen cyanide, a volatile compound produced by microbial antagonists, suppresses fungal inhibition (Michelsen and Stougaard, 2012). Cell wall degrading enzyme secretions also implicate antagonism towards plant pathogens (Zhang *et al*., 2012).

2,4-diacetylphloroglucinol (DAPG) is a polyketide antibiotic that is produced by *Pseudomonas* strains that predominantly belong to the *P. protegens*, and *P. corrugata* subgroups led to the degrading pathogen cell membrane (Mishra and Arora, 2012). Antibiotic DAPG, produced by P. *fluorescens* F113 exhibited inhibition ability towards *P. atrosepticum* (Cronin *et al*., 1997). *Pseudomonas brassicacearum* LBUM300 produced DAPG and HCN that are responsible against bacterial canker of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis* (Lanteigne *et al*., 2012; Paulin *et al*., 2017). In addition, endophytic *P. brassicacearum* strain capable of producing DAPG, pyrrolnitrin, HCN, dimethyl disulfide and dimethyl trisulfide inhibited *P. c.* subsp. *carotovorum* activity is previously reported (Aghdam *et al*., 2022). Pseudomonas strains produce Pyoluteorin as a phenolic polyketide (Nowak-Thompson *et al*., 1997). Moreover, as a halogenated aryl pyrrole, pyrrolnitrin is produced by different bacterial genera (Costa *et al*., 2009) with specified antifungal activity.

The primary antagonistic effect of *P. protegens* FD6 was due to the compounds 2,4-diacetylphloroglucinol and pyoluteorin, which were effective against soilborne fungi (Zhang *et al*., 2020). Ramette and co-workers (2011) proposed that Phl+ Plt+ fluorescent *Pseudomonas* strains belong to the species *protegens.* Although *P. protegens* Pf-5 (obtained from cotton rhizosphere) and CHA0 (obtained from suppressive soil) reference strains produce the typical secondary metabolites, including HCN, DAPG, pyrrolnitrin, pyoluteorin and siderophores (Haas and Defago, 2005), other *P. protegens* strains with different production patterns are reported. The *P. protegens* strain Os17 obtained from rice rhizosphere contains gene clusters for HCN, DAPG, and pyoverdine, but pyrrolnitrin and pyoluteorin gene clusters are absent (Takeuchi *et al*., 2015). Herein, the biosynthesis antibiotic cluster genes of *P. protegens* strains IrPs5, IrPs9 and IrPs10 are varied compared to reference strains CHA0 and Pf5.

Conclusion

The current study has shown that coated potato tubers with *P. protegens* strains IrPs8 and IrPs18 can decrease incipient infection of *P.*

carotovorum subsp. *carotovorum* under *in vivo* and storage conditions. Antimicrobial metabolites derived from *Pseudomonas* spp. are variable, making this genus a proper biocontrol agent in the belowground niche. The current study demonstrates the potential of *P. protegens* to mitigate soft rot symptoms caused by Pcc on potato tubers. These results should be validated under bulk storage conditions and then can be performed besides other available strategies.

Conflict of Interest: The authors declare no conflict of interest.

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توان کاهش پوسیدگی نرم سیب‌زمینی توسط جدایه‌های بومی *Pseudomonas protegens* در شرایط انباری

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چکیده: در میان باکتری‌های فرایشه، اثرات مفید باکتری‌های *Pseudomonas* در اکوسیستم‌های کشاورزی شناخته شده است. غربال‌گری سودومونادهای فلورسنت به‌دست آمده از فرایشه سیب‌زمینی منجر به انتخاب پنج عامل زیستی کنترل کننده پوسیدگی نرم ناشی از *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) (JX029052) شد. غربال‌گری تحت شرایط پتري ديش، در آزمون بيماري‌زايي روي غده‌های سالم سیب‌زمینی و با استفاده از تیمارهای پیشگیرانه و درمانی در انبار انجام گرفت. ویژگی‌های مهار زیستی مربوط به باکتری‌های فرایشه مورد بررسی نیز ارزیابی شدند. براساس آزمایش‌های فنوتیپی، جدایه‌های بازدارنده منتخب، به گروه سودومونادهای فلورسنت تعلق داشته و براساس توالی‌یابی ژن 16S rRNA در خوشه *P. protegens* قرار گرفتند. جدایه‌های IrPs8 و IrPs18 توانایی تولید سیدروفور، سیانید هیدروژن و پروتئاز، تشکیل بیوفیلم قوی را دارند و نیز حامل ژن‌های *phl*، *plt* و *prn* می‌باشند که به‌ترتیب با تولید ۲، ٤-دی‌استیل‌فلوروگلوسینول، پیولوتئورین و پیرول‌نیترین مرتبط است. غده‌های تیمار شده با جدایه‌های IrPs8 و IrPs18 ، میزان پوسیدگی نرم ایجاد شده با Pcc را به‌ترتیب به میزان ٦۳/٤ و ٦٥/٥ درصد در آزمایش‌های پیشگیرانه و به میزان ٥۷/۸ و ٥۸/۳ درصد در آزمایش‌های درمانی در شرایط انباری کاهش دادند که با نتایج *in vivo* همخوانی دارد. این مطالعه روي توان بالقوه جدایه‌های فرایشه *P. protegens* به‌عنوان باکتری‌های مفید تأکید دارد که قادرند به‌عنوان پوشش‌های حفاظتي برای غده‌های سیب‌زمینی در شرایط انباري پیشنهاد شوند.

واژگان کلیدی: ژن‌های بیوسنتز آنتی‌بیوتیک، مهارزیستی، *Pectobacterium carotovorum* subsp. *carotovorum*، لهیدگی غده