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Weeds Associated Bacterial Endophyte Producing Pantocin against *Pectobacterium carotovorum* subsp. *carotovorum*

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ABSTRACT

In the present study, bacterial endophytes were obtained from weeds of potato fields. Their antagonistic activity was screened against potato storage pathogen, *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) (JX029052), in the maceration assay. An endophytic strain, PC-2B was isolated from *Convolvulus arvensis* L. as a dominant weed of potato fields. In vivo application of this strain led to 58.8% reduction in tuber decay. This motile strain that can produce amylase was identified as *Pantoea* sp. using phenotypic features and 16S rRNA sequencing. Following PC-2B treatment, 56.7% Reduction in Disease Incidence (RDI) was obtained in preventative and 52% in curative challenges under semi-practical storage trails. Anti-Pcc bioactive compounds of *Pantoea* sp. was extracted and identified based on H NMR and FT-IR techniques. Two peptide antibiotics including Pantocin A and B with inhibitory effect against Pcc were characterized. These results might indicate that the tested *Pantoea* strain could be a promising candidate to protect potato tubers against soft rot disease caused by *Pcc.* However, large-scale complementary trials have to be conducted to validate these results before any recommendations.

Keywords: *Convolvulus arvensis* L., FT-IR technique, H NMR technique, Potato decay, Tuber maceration.

INTRODUCTION

Pectobacterium carotovorum subsp. *carotovorum* (*Pcc*) is one of the major bacterial pathogens of potato causing tuber maceration (Czarkowski *et al*., 2015). This subspecies has been reported to be a common bacterial pathogen causing soft decay in potato stores of Iran (Baghaee-Ravari *et al*., 2013). Latently, infected tubers were considered as a primary source of infection and tuber contamination can occur in different phases during potato harvest, transportation, and storage (Toth *et al*., 2003). In potato production, applying usual integrated methods including usage of *Pectobacterium* free tubers, inhibition of tuber wounding and hygienic practices lead to partial protection towards soft rot disease (Czajkowski *et al*., 2011). In addition, application of beneficial microbial microorganisms has been recommended for complementation of common management approaches (Krzyzanowska *et al.,* 2019).

Jiang *et al*. (2019) reported that several factors including plant health, developmental stage, soil type, climate change, and pesticide treatment could affect the structure pattern of bacterial communities related to the plant. Endophytic bacteria are able to establish symbiotic relationship within internal tissues of plants without causing any adverse effects (Bakker *et al*., 2013). These valuable microorganisms provide better survival advantages to their host plants, as they

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enhance plant growth (Santoyo *et al*., 2016), modulate plant defense (Ryan *et al*., 2008; Dini-Andreote, 2020) and suppress phyto-pathogen growth and survival through secretion of biologically active molecules (Compant *et al*., 2010). Since the ecological niche occupied by microorganisms showing endophytic lifestyles is the same as the plant pathogens, they can be considered as a proper option for controlling plant diseases (Morelli *et al*., 2020).

There are different kinds of weed host in the fields of agronomic crops. Weeds are easily found in nature, well adapted to the environment and usually known as inhibitors of germination or growth in agronomic plants, leading to substantial crop yield reduction (Sturz *et al*., 2001; Blanco, 2016). Plant vigor and resistance to pathogens could be related to the associated microorganisms (Catambacan *et al*., 2021). It is reported that weeds showing positive interaction with different microbial groups in comparison to crops that make them a better competitor (Massenssini *et al*., 2014). The presence of these beneficial endophytes may contribute to plant survival, growth, and antagonism against pathogens (Strobel *et al.,* 2004).

Although a number of endophytes showing anti-*Pectobacterium* activity have been obtained from potato plants (Pavlo *et al.,* 2011; Pagani *et al*., 2014; Azaeiz *et al*., 2018; Ha *et al*., 2018, Mohammad-nejad Aghdam *et al*., 2022), only one research has initially reported the efficacy of weed endophytic bacteria towards this soft rot agent (Krimi *et al*., 2016).

The aim of the current study was to isolate native endophytes from weeds of potato fields in Iran and to determine their antagonistic activity against the soft rot pathogen, *Pcc in vitro* and under storage conditions. The bioactive compounds of the representative endophyte, *Pantoea* sp. PC-2B (GenBank accession no. OL589315) with inhibitory effect against *Pcc* were further characterized using Nuclear Magnetic Resonance (H NMR) and Fourier Transform Infrared Resonance (FT-IR).

MATERIALS AND METHODS

Endophyte Isolation from Weeds of Potato Fields

Dominant weeds from potato fields of Ardabil, Hamadan, Isfahan, Kerman and Khorasan Razavi provinces in Iran including *Convolvulus arvensis* L., *Solanum nigrum* L., *Amaranthus spinosus* L.*,* and *Chenopodium album* L. were collected and identified by the Herbarium of Research Center for Plant Sciences, Ferdowsi University of Mashhad, Iran. Healthy root segments were washed under running tap water, immersed in sodium hypochlorite 1% (5 minutes) and 70% ethanol (1 minute) and washed three times with Sterile Distilled Water (SDW). After the disinfection, the samples were ground in a sterile mortar containing SDW and plated onto the Nutrient Agar (NA) for 5 days at 28°C. Validation of sterility was checked by spreading the last wash water and pressing the surface tissues on the NA plates in order to evaluate any microbial contamination. Selection of the strains was performed based on type of bacterial colony for further studies.

Screening for Antibacterial Activity on Agar Plates

The antibacterial potential of endophytes was evaluated towards native pathogenic strain, *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) (JX029052) (Baghaee-Ravari *et al*., 2013). Bacterial suspension of each endophytic strain (15 µL) was spotted on the surface of NA plates and incubated at 28°C. After 24 hours, *Pcc* suspension with the concentration of 10^8 CFU mL^{-1} was sprayed on the treated plates with three replicates. The inhibition zone around the bacterial colony was considered as positive antagonistic activity and measured following 48 hours.

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In vivo Antagonistic Activity against *Pcc* in the Maceration Test

One endophytic strain (PC-2B) that showed the inhibition diameter of 21 mm towards Pcc was further evaluated in tuber soft rot assay using intact potato tubers according to literature with minor modification (Zhao *et al.,* 2013). Disinfection of tubers was done with 5% sodium hypochlorite. Bacterial suspension of endophyte was injected at a depth of 1.5 cm in seven potato tubers. After 2 hours, each wound was filled with *Pcc* suspension (20 µL) and the tested potatoes were stored at 25°C for 72 hours. Finally, the rotted tissue was removed by scooping out and weighted. Treated tubers individually inoculated with *Pcc*, sterile water, and antagonistic strain were considered as positive and negative controls, respectively.

Phenotypic and Molecular Identification of PC-2B

For bacterial identification of endophyte, biochemical tests were carried out according to the method described by Schaad *et al.* (2001). Moreover, 16S ribosomal RNA gene sequencing was performed after amplification with 27f and 1492r universal primers (Bredow *et al*., 2015).

The PCR products were purified by the QIAquick PCR purification kit (Qiagen, USA) and sequenced in both directions using an automatic sequencer 3730X (Macrogen, Korea). The obtained sequence was aligned with the corresponding sequences retrieved from NCBI GenBank database using ClustalX 1.81 (Thompson *et al.,* 1997). The Maximum likelihood tree was drown using MEGA 6 software (Tamura *et al.,* 2013).

Screening for Biofilm Formation, Motility and Enzymatic Activities of Strain PC-2B

The formation of biofilm in PC-2B strain was surveyed based on O'Toole and Kolter (1998). A 24 hour endophytic culture in Luria Bertani medium was added to the 96 well microtiter plate with six repeats. Following 48 hours incubation, bacterial cells, which were adhered tightly to the well, was strained with crystal violet (0.1%). Subsequently, to solubilize the used dye, 200 µL of 99% ethanol was added to each well, and the amount of the solubilized dye was quantified by measuring the absorbance at 570 nm using a microplate reader.

In order to check the antagonist mobilization, 24 h-old culture of PC-2B strain was spotted on the middle of the minimal swim motility agar plates (Anderson et al., 2003). The treated plates were kept at 28°C and, after 24 hours, the diameter of bacterial colony was measured in four replications.

Moreover, the enzymatic potency of four enzymes in PC-2B strain including protease (Smibert and Krieg, 1994), pectinase (Jayasankar and Graham, 1970), amylase and cellulase (Hankin and Anagnostakis, 1977) was estimated.

Semi-Practical Storage Trail for Evaluation of Endophytic Strain against *Pcc* Infection

In two separate experiments (preventative and curative assays) with seven repeats, sterilized potato tubers, as described earlier, were used. At first, two wounds were made in the sterilized tubers using sterile borer. In the preventative test, tubers were floated in antagonist suspension for 20 minutes. Then, Pcc suspension (15 µL, 10^8 cell forming Unit of bacteria (CFU)/mL) was inoculated to each wound. To investigate the curative assay, 12 hours after potato infection, the tubers were dipped in the antagonist suspension as mentioned above.

In the control treatments, sterile water replaced the antagonist suspension in both assays. At the end of the tests, the calculation of RDI (Reduction in Disease Incidence) index and pathogen Penetration (P) was estimated according to Sameza *et al*.

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(2016) and Lapwood *et al*. (1984), respectively.

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

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

D: decay diameter (mm) ; d: decay depth (mm); P: penetration

Isolation and Purification of Secondary Metabolites from Strain PC-2B

Pure bacterial colony was cultured in 400 mL of nutrient broth in 1,000 mL Erlenmeyer glass flask and shaken at 200 rpm at 28°C for 7 days. The bacterial suspension was centrifuged (8,000×g at 4°C for 10 minutes) and the supernatant was collected into a measuring cylinder, acidified to pH≤ 2 by ethyl acetate in a 1:1 ratio (v/v). After shaking for 30 minutes at 120 rpm, the resulting mixture was extracted with ethyl acetate (3×200 mL). The organic phase was dried and removed by anhydrous sodium sulfate and a rotary evaporator, respectively. The resulting oily liquid was dissolved in methanol and loaded onto the pre coated silica gel plates to fractionate using chloroform/methanol (20:1, v/v) as mobile phase. Separated spots were visualized by UV light (254 nm). The resulting crude obtained fractions were washed with petroleum ether and di ethyl ether, successively, in order to evaluate their antibacterial activities.

Characterization of Purified Substances with Inhibitory Effect towards *Pcc*

The isolated fractions that exhibited zone of inhibition against *Pcc* were characterized thoroughly by ^{1}H NMR, FT-IR, and Mass spectroscopies. Therefore, Fourier Transform Infrared spectrum (FT-IR) was obtained on Avatar 370 FT-IR Thermo Nicolet to detect the indicated functional groups on the organic product. The Nuclear Magnetic Resonance spectrum (^{1}H NMR, 300 MHz) was recorded on a Bruker Avance-III 300 NMR Fourier transformer spectrometer. Tetramethylsilane (TMS) was used as internal standard and the chemical shifts were reported in ppm.

RESULTS

Antibacterial Activity of Endophyte Strain Isolated from Potato Weeds

At the end of an incubation period in antibiosis assay, the inhibition zones as a criterion for antibacterial potency was measured in millimeter. Among 38 bacterial strains isolated from root of different potato weeds, six strains showed weak inhibition halo in the range of 0.2-0.3 mm. Only one strain (PC-2B) obtained from *Convolvulus arvensis* L., exhibited antagonistic ability (21 mm) against *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) (Figure 1) and was selected for further studies.

In Vivo Maceration Assay of Potato Tubers at the Presence of Endophytic Strain

To determine the mean value of tuber maceration, rotting tissue was measured following 72 h in both control and treated tubers. Application of endophytic strain, PC-2B led to reduction in tuber decay by 58.8%. The weight of macerated tissue was recorded 2.1 g/wound compared with the positive control, which was inoculated only by Pcc (JX029052) as shown in Figure 2. Under this *in vivo* trail, the tested antagonist (PC-2B) had no ability to cause tuber maceration symptoms.

Phenotypic and Molecular Characterization of Endophyte PC-2B

PC-2B stain was rod-shaped bacterium that showed negative reaction in Gram, oxidase, urease and H_2S production tests. This strain

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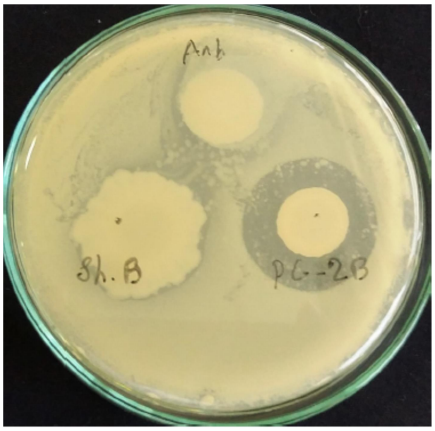

Figure 1. Antibacterial potency of *Convolvulus arvensis* L. endophytic strain (PC-2B) against postharvest soft rot agent, *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*-JX029052) in the plate assay following 2 days.

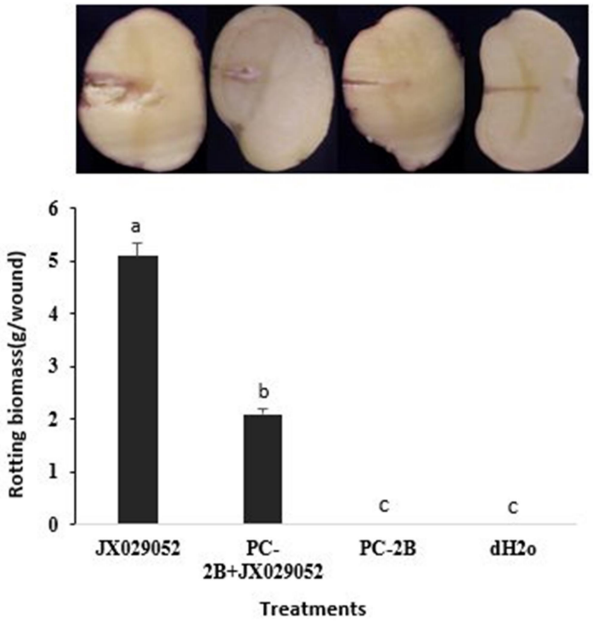

Figure 2. In vivo potential of endophytic strain (PC-2B) individually or in combination with Pcc-JX029052 (PC-2B+JX029052) were compared with positive (JX029052) and negative (dH_2O: Distilled water) controls in potato tuber maceration assay after 72 hours with seven repetitions ($P \leq 0.05$).

also showed no growth at 37ºC and had positive reaction in catalase assay

and oxidative/fermentative metabolism of glucose. Moreover, the partial 16S rRNA sequence from PC-2B stain was analyzed and deposited under the accession number of OL589315 in GenBank. In order to understand the relationship of endophyte PC-2B with other related sequences retrieved from GenBank, a phylogenetic tree was generated using maximum likelihood method with 1000 bootstrap replicates (Figure 3). PC-2B strain showed a high degree of identity (99%) to endophytic *Pantoea* strain from mulberry (MH768997). Its partial 16S rRNA gene sequence was deposited under the accession number OL589315 in GenBank and the current strain was designated as *Pantoea* sp.

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Further complementary studies are needed for definitive identification.

Biofilm Formation, Mobilization and Secretion of Lytic Enzymes by the Antagonistic Strain

Biofilm biomass volume was quantified by crystal violet staining. The value of the crystal violet dye intensity adheres to biofilm cells measured as 1.6 at 550 nm using a plate reader. PC-2B strain formed colony on minimal swim motility agar plates by the diameter of 13 mm. In addition, the enzyme degradation halo was only observed in amylase plate using the tested antagonist.

Protection of Potato Tuber against Soft Rot Development under Storage Conditions

When the endophyte was used before and after the pathogen inoculation, two factors consisting of Reduction in Disease Incidence (RDI) and depth of *Pcc* Penetration (P) in treated potato tubers were estimated. The tissue rot development was recorded 8.25 mm in *Pcc*-inoculated tubers after one week, while application of endophyte reduced the depth of *Pcc* extension in preventative and curative assays by 2.3 and 3.1 mm, respectively. Moreover, the percentage of RDI was obtained 56.7% in preventative (Figure 4) and 52% in curative tests.

Detection and Structural Elucidation of Pantocin A and B in *Pantoea* sp.

Following extraction of secondary metabolites from *Pantoea* sp., two dominant fractions were subjected to H NMR and FT-IR methods. Two antibiotics including Pantocin A (Figure 5) and B (Figure 6) were detected, both of which generated inhibition

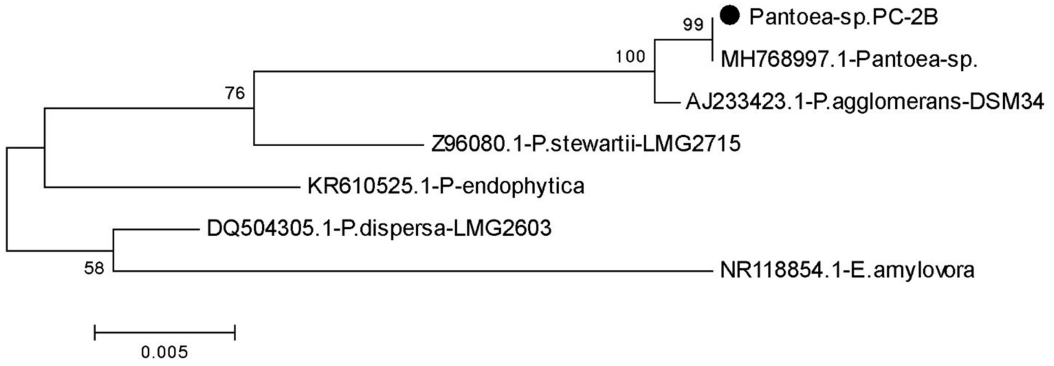

Figure 3. Maximum Likelihood phylogenetic tree based on partial 16S rRNA gene sequence of the endophyte (PC-2B) related to *Convolvulus arvensis* L. (potato weed) from Iran (●) and other Pantoea strains. Numbers at the branches denote the bootstrap values (based on 1,000 replicates).

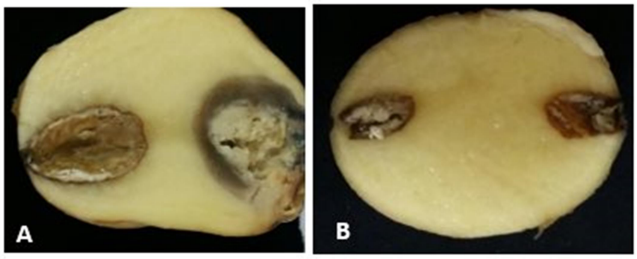

Figure 4. Effect of the bacterial endophyte (PC-2B) isolated from *Convolvulus arvensis* L. on potato soft rot development in preventative assay under storage conditions. A: *Pcc*-JX029052; B: *Pantoea* sp. (PC-2B).

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zones against the soft rot pathogen (*Pcc*) in plate assay.

Characterization of Pantocin A:

Yellow; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.59 – 2.63 (m, 2H), 2.78 (d, *J*= 6.2 Hz, 1H), 2.84 (d, *J*= 2.6 Hz, 1H), 3.28 (s, 1H), 3.54 (s, 1H), 4.27 (s, 1H), 5.69 (s, 1H), 5.94 (s, 1H), 6.55 (s, 1H), 7.05 (s, 2H), 8.31 (s, 1H), 8.74 (s, 2H), 11.76 (s, 1H). (Figure 5) IR (KBr disc, cm^{-1}) ν 3369 (NH), 2957, 2925, 2854 (CH), 1665 (C=O), 1566, 1246 (C-O); MS (*m/z*): 308 (M^+).

Characterization of Pantocin B:

Yellow; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.10 (s, 3H), 2.70 (s, 2H), 3.02 (d, *J* = 7.3 Hz, 2H), 3.76 (s, 1H), 4.00 (s, 2H), 6.73 (s, 2H), 8.07 (s, 2H). (Figure 6) IR (KBr disc, cm^{-1}) ν 3340 (OH), 3056 (NH), 2958, 2928, 2876, 1711, 1677; MS (*m/z*): 173 (M^+-$C_2H_5NH_2$), 147 (M^+-$C_2H_5CO_2H$), 128 (M^+-CO_2, $C_2H_5NH_2$).

DISCUSSION

Different endophytic microorganisms are reported to be distributed in a wide range of host plants (Rosenblueth and Martínez-Romero, 2006). Weeds are abundant in nature, thrive in diverse ecosystems, and can host several microorganisms such as endophytes (Amith *et al*., 2019). It seems that weed-associated microorganism affect different functions of weeds and could be

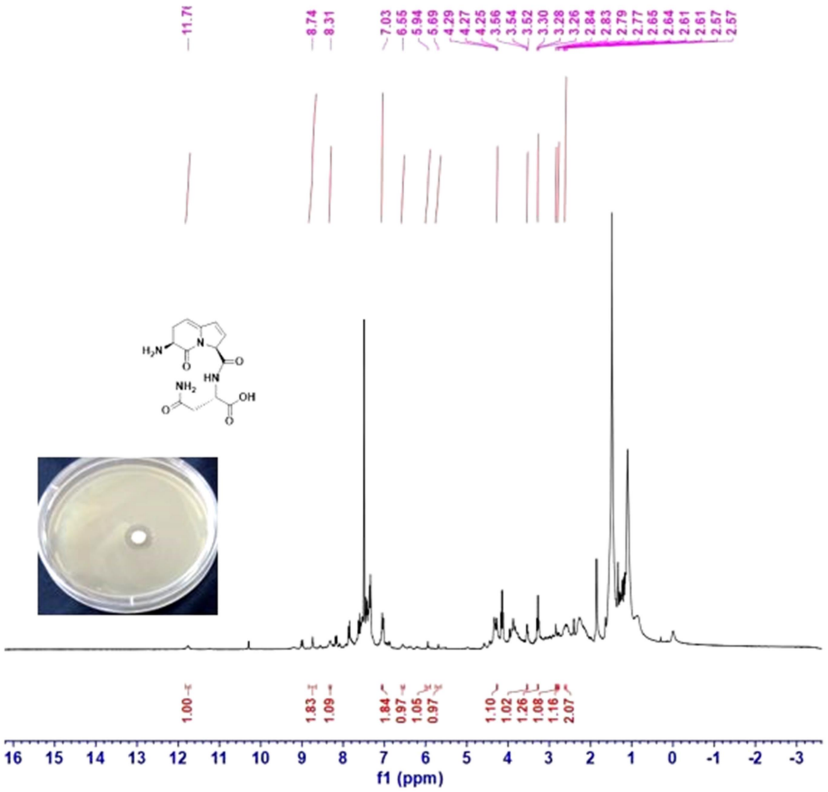

Figure 5. The ^{1}H NMR spectrum of Pantocin A extracted from *Pantoea* sp. PC-2B strain, and its anti-*Pcc* potency towards *Pectobacterium carotovorum* subsp. *carotovorum*, (plate figure).

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transmitted from weeds to other plants by several ways (Massenssini *et al*., 2014). The cultivable endophytes obtained from weeds were reported as biocontrol agents against fungal (Catambacan *et al*., 2021) and bacterial (Krimi *et al*., 2016) diseases.

Bio-Control Agents (BCAs), especially endophytes, are considered as a sustainable ecological alternative for decreasing the use of agricultural chemicals and management of the diseases. Although the usage of microbial genera including *Bacillus*, *Pseudomonas* and *Trichoderma* are common as BCAs (Chen *et al*., 2020; Erazo *et al*., 2021), some beneficial *Pantoea* strains can counteract the growth of plant pathogens (Asis *et al*., 2004; Jiang *et al*., 2019). Some formulations of *Pantoea* strains such as BlightBan C9-1™ and BloomTime Biological™, are commercially applied as biocontrol products and used instead of antibiotics in fire blight management (Smits *et al*., 2019).

While the competitive exclusion is the main function of bio-control *Pantoea* strains towards plant pathogens (Smits *et al.,* 2011), some strains can produce peptide antibiotics as an inhibition mechanism (Pusey *et al*., 2011; Kamber *et al*., 2012). There are reports indicating that the bacteria of the *Pantoea* genus can secrete natural compounds including herbicolins (Ishimaru *et al*. 1988), pantocins (Wright *et al*., 2001) and some unknown antibiotics (Pusey *et al*., 2008).

Among antagonists of *Erwinia amylovora*, the bacteria including *Pantoea vagans* C9-1 and *Pantoea* sp. Eh252 produced Pantocin A (Vanneste *et al*., 2008; Ishimaru *et al*., 2017) and *P. agglomerans* Eh318 produced Pantocin A and B (Wright *et al*., 2001). Pantocin A and B blocks the function of L-histidinol phosphate aminotransferase (Jin *et al*., 2003) and N-acetylornithine transaminase (Brady *et al*., 1999) in the pathway of L-histidine and arginine, respectively.

The inhibitory effect of *P. agglomerans* and *Pantoea* sp. isolated from potato against *Pectobacterium atrosepticum* (Sturz and Matheson, 1996) and *Giberella pulicaris* (Schisler and Slininger, 1994) is previously reported, respectively. However, no work until now has assessed the antibacterial potential of *Pantoea* sp. isolated from weeds of potato towards *Pcc*.

In this study, just one endophytic strain (PC-2B) from *Convolvulus arvensis* L.*,* collected from potato field, reduced the soft rot decay of potato tubers by 58.8%. According to some phenotypic features and 16S rRNA gene sequencing, the tested strain belonged to *Pantoea* sp. Under storage conditions, use of PC-2B reduced soft decay incidence by 56.7 and 52% in preventative and curative trial, respectively, in comparison with the control. Yellow-pigmented motile endophytic strain produced amylase among lytic enzymes and also bioactive compounds including Pantocin A and B that was confirmed using H NMR, FT-IR techniques.

It seems that the competitive strategies of weeds in comparison to crops may be partly due to the presence of endophytes and their related metabolites (Strobel *et al.,* 2004). This suggests the application of weed endophytes as alternative agents against pathogen of crops. In conclusion, production of peptide antibiotics including Pantocin A and B by a weed endophytic strain of *Pantoea* sp. as a biological control agent of *Pectobacterium carotovorum* subsp. *carotovorum*-JX029052 is reported here for the first time. Further research is needed in order to validate applying this endophyte during storage in large scale with respect to environmental, health, and safety considerations**.**

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اندوفیت باکتریایی مرتبط با علف هرز تولید کننده پانتوسین علیه *Pectobacterium carotovorum* subsp. *carotovorum*

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چکیده

در تحقیق حاضر، اندوفیت های باکتریایی از علف های هرز مزارع سیب زمینی جداسازی شدند. فعالیت آنتاگونیستی آنها در برابر بیمارگر انباری سیب زمینی، *Pectobacterium carotovorum* subsp. *carotovorum* در آزمون سنجش لهیدگی غده بررسی شد. جدایه اندوفیت PC-2B از *Convolvulus arvensis* به عنوان علف هرز غالب مزارع سیب زمینی با هاله بازدارندگی ۲۱ میلی متر در غربالگری اولیه انتخاب شد. کاربرد این جدایه در شرایط درون شیشه، منجر به کاهش ۵۸/۸ درصدی پوسیدگی غده گردید. این جدایه متحرک تولید کننده آمیلاز بر اساس ویژگی های فنوتیپی و توالی یابی ژن ۱۶ S rRNAبه عنوان Pantoea sp. مشخص شد. در شرایط انبارداری شبه عملی، پس از تیمارPC-2B ، کاهش وقوع بیماری در آزمون هاي پیشگیرانه و درماني به ترتیب ۵۶/۷ و ۵۲ درصد بدست آمد. ترکیبات فعال زیستي ضد Pcc از باکتری Pantoea sp. استخراج و با بکارگیری روشهای H NMR و FT-IR شناسایی شدند. دو آنتی بیوتیک پپتیدی شامل پانتوسین A و B با اثر بازدارندگی در برابر Pcc مشخص شدند. جدایه باکتری مورد آزمایش ممکن است کاندیدای قابل قبولی برای محافظت از غده¬های سیب زمینی در برابر پوسیدگی نرم باشد. با این وجود، این نتایج باید از طریق آزمایشات تکمیلی در مقیاس بزرگ قبل از ارائه هر گونه توصیه، تأیید شوند.

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