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Quantification of Pathogenicity Genes Expression in *Pectobacterium carotovorum* by qRT-PCR

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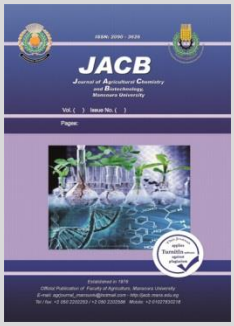

ABSTRACT

Bacterial soft rot disease is one of the most important and widespread bacterial diseases that cause destructive losses to the crop in a matter of days. The disease causes severe many vegetable crops including potato. In this study, four isolates of *Pectobacterium carotovorum* the main causal of the soft rot disease were isolated from naturally rotted tubers. Then, the isolates were subjected to a new set of primer pairs designed to amplify the *pel* gene which distinguish the *P. carotovorum* strains. The results confirmed that four isolates belong to *P. carotovorum* by having the *pel* gene. Then, a pathogenicity test was performed on potato tubers which showed variability between the isolates in pathogenicity and diseases severity. Finally, a quantitative reverse transcriptase PCR (qRT-PCR) technique was used to investigate the effect of the presence of the potatoes tissues on stimulating the expression of the pathogenicity genes. The gene expression of five pathogenicity genes was studied included: polygalacturonase (*Peh*), pectate lyase (*Pel*), xylanase (*Xy*), protease (*Prt*), and cellulase (*Cel*). The results confirmed that the presence of potatoes tissues stimulates the expression of pathogenicity genes in both strong and weak *P. carotovorum* isolates.

Keywords: *P. carotovorum* , qRT-PCR, Polygalacturonase, pectate lyase – cellulase, xylanase, protease

INTRODUCTION

Globally, potato is one of the most important food after wheat, rice and maize (Hawkes, 1992). In the field and during storage, many bacterial and fungal diseases can attack the tubers (Wilson, 1960). *Pectobacterium*, certain *Pseudomonas* spp. and *Bacillus* spp are the mainly pathogens of potato (Lelliott *et al*., 1966). *P. carotovorum* subs*P. carotovorum* is a plant-specific pathogen that cause general plant tissue maceration. The production of plant cell wall degrading enzymes is the main virulence factor of this pathogen. It is producing enzymes, such as: Polygalacturonase, pectate lyase, xylanase, cellulases and proteases, that degrade the plant cell wall (Pérombelon, 2002 and Thomson *et al*., 1999).

Sequencing of several genomes of *P. carotovorum* subs*P. carotovorum* were obtained (Smits *et al*., 2010; Park *et al*., 2011; and Powney *et al*., 2011). The genomes size ranged from 3.8 to 5.1 Mbp, with coding regions accounted for about 86 % of the total genome, and about 4,263 annotated coding sequences (CDSs) with a length of about 978 bp. The sequencing data confirmed the presence of PCWDEs genes and other pathogenic determinants.

Virulence factors genes like, the producing of extracellular enzymes and others including quorum sensing system, flagellar and chemotactic, type II and III secretion systems genes were highly conserved in the *Pectobacterium* strains (Li *et al.* 2019)

Real time-PCR is reliable tool that have been applied to many biological processes for molecular detection and quantification (Chin *et al*., 2008 and Holmes *et al*., 2006). Since qPCR became available in the 1990s, it has been applied to many different aspects (Zhang and Fang, 2006; N'Guessan *et al*., 2010 and Sharp *et al*., 2007).

Targeting functional genes and measuring its gene expression are performed via qRT-PCR, by converting isolated RNA to complementary DNA (cDNA) then quantifying the PCR-amplified DNA (Li *et al*., 2010).

Recently, qPCR was used on a wide scale to detect viral and bacterial causing potato diseases and estimate the occurrence of some target pathogens (Malko *et al*., 2019). The analysis of infected leaves and tubers confirmed the dominance of certain potato viruses, *Clavibacter michiganensis* subsp. *sepedonicus, P. carotovorum* subs*P. carotovorum* , and *Pectobacterium atrosepticum* in most European regions as Russia.

Ahmed *et al.* (2017) used the qPCR technique to quantify the *P. carotovorum* bactericidal activity and to evaluate the growth of *P. carotovorum* under the effect of potassium tetraborate tetrahydrate.

Due to the importance *P. carotovorum* in causing an aggressive disease to Potatoes, this investigation was designed to better understand the genetic background of *P. carotovorum* the main causal of the bacterial soft rot disease and to investigate the effect of the presence of potato tissues on stimulating the expression of the pathogenicity genes.

MATERIALS AND METHODS

Isolation of the causal bacteria.

Isolation of causal pathogen was carried out from naturally infected potato tubers, with soft rot symptoms collected from Assiut Governorate during 2013-2014 seasons. The infected samples were washed with tab water several times, then the surface was sterilized using in 1%

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sodium hypochlorite for two minutes and rinsed twice with water. Then, a small part of the diseased tissues was macerated in 5 ml of 0.05 M potassium phosphate buffer and a loopful of the suspension was streaked onto nutrient sucrose agar medium (Dowson, 1957) and incubated at 28°C for 48 h. Single colony of the isolates was sub-cultured onto slants medium and maintained at 4ºC for further studies.

Pathogenicity tests.

Isolated bacteria were tested for its ability to cause soft rot disease in both slices (McGuire and Kelman, 1984) and on the full tubers (Yaganza *et al*., 2003). Four replicates were used for each tested isolate. And all the data were analyzed statistically using L.S.D. test (Gomez and Gomez, 1984).

For the Pathogenicity test on slices, one-centimeter-thick of a sterilized potato slice were kept on a filter paper in Petri-dishe and a loopful of the tested isolates (24 hr. old) was streaked over the slice and incubated at 27°C and observed daily for rotting over a period of 3 days (McGuire and Kelman, 1984).

The pathogenic capability of the tested isolates on the whole tuber were performed by creating a cavity (about 1 cm in depth and 0.5 cm in width) in each tuber by a cork-borer. Then 100 µl of an overnight culture of the tested bacterial isolate were placed in the bottom of the potato cavity. Treated tubers were kept in clean sterilized plastic containers supplemented with a sterilized moist cotton and incubated at 27°C for 4 days. Rotted tubers were weighted and the rotted tissues were removed and weighted again. (Yaganza *et al*., 2003).

Expression and quantification of the degrading enzymes by qRT-PCR.

qRT-PCR experiment was performed to understand the role of degrading enzyme genes in the pathogenic mechanism of *P. carotovorum* by quantifying of the expression of these genes and whether or not the presence of potato tissue in bacterial cultures stimulates expression of pathogenic genes. The expression of several pathogenicity genes was studied included: polygalacturonase (*Peh*), pectate lyase (*Pel*), xylanase (*Xy*), cellulase (*Cel*), and protease (*Prt*). Two isolates, differ in their disease severity, were selected to be used in this study. Isolates were tested under both control (without potato tissue) and induced conditions (potatoes tissues were added to the bacterial cultures)**.**

New sets of primers were designed according to the sequences of the candidate pathogenicity genes (*Peh, Pel, Cel, Xy,* and *Prt*) obtained from the GenBank (https://www. ncbi. nlm.nih.gov/). Sequences of the used primers are listed in Table 1.

Table 1. Primer sequences and codes used in qRT- PCR quantification.

Gene name	Primer code	Primer Sequence	Band size	Tm
Polygalacturonase (*Peh*)	Peh-Fw	TCGTGGTTGAAGGGGGAACG	277	56
	Peh-Rv	CCGTTGCCATCAATCGTGCC		57
Pectate lyase (*Pel*)	Pel2-Fw	GTCGCTTCTTGCAGGTCTGG	1661	58
	Pel2-Rv	CGCCAACGTTCAGCAGGAAG		58
Cellulase (*Cel*)	Cel-Fw	GGGTGCAACTGAGAGGGATC	215	55
	Cel-Rv	GATGATGTAGACGCCGAGGC		55
Xylanase (*Xy*)	Xy-Fw	CGCTCAGCGACCACATCAAC	500	57
	Xy-Rv	CTCATCGGTGGTGAGTTGGC		55
Protease (*Prt*)	Prt-Fw	CTGAAGGACGACACCAACGC	356	56
	Prt-Rv	ATGGACAGCACACGTGGCAG		58

Total RNA was extracted from three days old bacterial liquid cultures using SV Total RNA Isolation System (Promega) and by following the manufacturer's instructions. Then a DNase treatment using the Max kit from Qiagen was applied to remove residual genomic DNA. RNA concentration and purity were measured in Nano Drop Spectrophotometer ND1000 (Nano Drop Technologies). After that, all RNA samples were diluted to a final concentration of 50 ng/µL and a Reverse transcription for the RNA samples were performed with the first-strand cDNA Synthesis Kit (Amersham Biosciences, GE Healthcare) using pd (N) random hexamers primers.

The qRT-PCR was performed at the Molecular Biology Research Center (MBRU), Assiut University in a Bio-Rad iCycler. The qRT-PCR master mix for each gene was prepared by the following reaction components: 12.5µl iQ SYBR Green Super Mix (Invitrogen), 0.25µl of each primer (10µM), and 8µl nuclease-free water. Then, 4µl of the cDNA were added to a 21µl of the master mix. The following PCR protocol was used: initial denaturation (95°C for 10 minutes), followed by repeated 40 cycles (denaturation: 95°C for 15 sec, annealing: 60°C for 20 sec, 72°C for 60 sec with a single fluorescence measurement), followed by 72°C for 7 min and then cooling to 4°C.

For quantification of gene expression, the cycle threshold (Ct) was determined for each gene transcript. The Relative Quantification (ΔΔCT) method was utilized to calculate fold change (Schmittgen and Livak, 2008). The gene expression of all tested genes was normalized with the absolute expression mean values of the reference gene (*dspE*) (Marquez-Villavicencio, *et al*. 2011).

RESULTS AND DISCUSSION

The pathogenicity of the isolates

Four bacterial pure isolates were obtained and tested for its Pathogenicity both potato tubers and slices. All the tested isolates were able to cause maceration of potato slices after 24 hours from inoculation, but they varied in their Disease severity. Data presented in Table (2) indicated that isolate ASY. 7 caused the highest diseases severity of rotted tissues (64.3%) followed by isolates ASY.33 (42.6%) and isolates ASY.26 (34.43%) which caused intermediate disease severity, then isolate ASY.26 was the lowest in disease severity (20.70%).

Table 2. The Disease severity percent of rotted potato tissues caused by the tested isolates

Isolate code	Disease severity (%)	L.S.D Category
ASY 7	64.30	A
ASY 26	34.43	B
ASY 33	42.60	B
ASY 34	20.70	C

Expression and quantification of degrading enzymes genes by qRT-PCR

The qRT-PCR experiment was performed to understand the role of degrading enzyme genes in the pathogenic mechanism of *P. carotovorum* by quantifying of the expression of these genes. And to test whether the presence of potato tissue in bacterial cultures stimulates expression of pathogenic genes or not. 2 isolates were used in this study (isolates ASY 7, and ASY 34). These two isolates were selected because they significantly differ in their disease

severity, since isolate ASY 7 caused the highest diseases severity of rotted tissues while isolate ASY34 showed the lowest diseases severity among the four *P. carotovorum* isolates (Table 2).

Five candidate pathogenicity genes (*Peh, Pel, Cel, Xy, and Prt*) were considered for the gene expression analysis using new designed sets of primers. The expressions of these genes were tested under both control (without potato tissue) and induced conditions (potato tissues were added to the bacterial cultures). All experiments were carried out in duplicates. The amplification and melting curves of qRT-PCR of *Peh*, *Pel*, *Cel*, *Xy*, and *Prt* genes are represented in Figure (1). The melting–curve analysis was done to confirm specific amplification. The melting–curve results for *Cel* and *Prt* genes, showed a single peak profile. This means that no unspecific product was detectable (Figure 1). A few less-sharp peaks in some samples appeared in *Peh*, *Pel* and *Xy* genes which means that they may contain unspecific products, so these contaminated samples were removed and not considered in the statistical analysis.

A relative quantification method calculating the fold difference of a target gene versus a reference gene (*dspE* gene) were used in all the comparisons according to Pfaffl (2004).

A comparison of the expression fold change of the five genes between the controlled and the induced conditions were made in the isolates ASY7 and ASY34 (Figure 2).

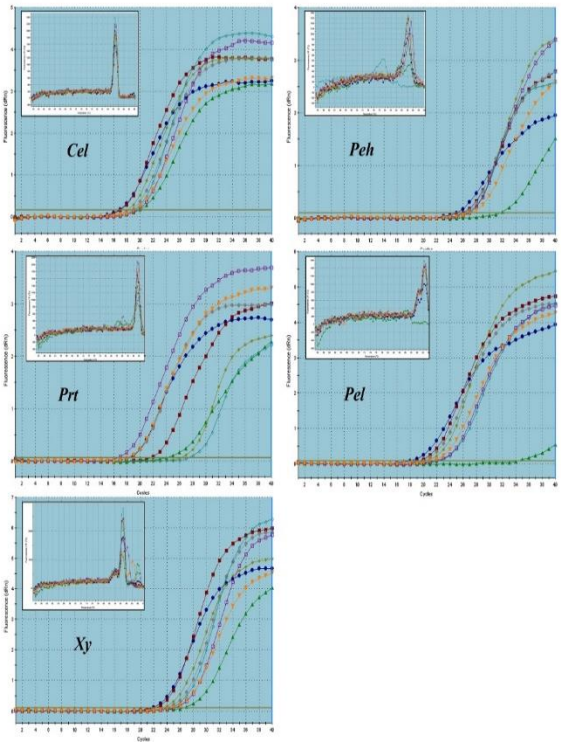

Figure 1. Amplification and melting curves for *Peh, Pel, Cel, Xy* and *Prt* genes obtained by qRT-PCR.

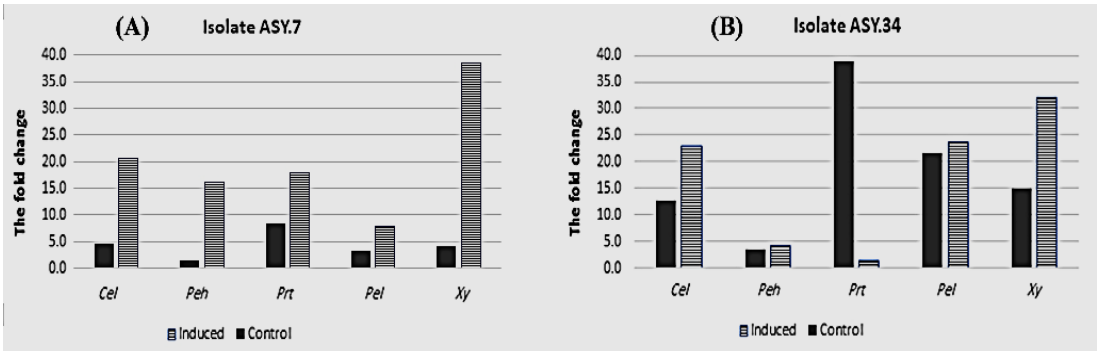

Figure 2. Expression fold change of the different genes showing a comparison between controlled and induced conditions in both (A) isolate ASY 7 and (B) isolate ASY 34.

Isolate ASY 7 showed a higher gene expression levels under the induced conditions than the control treatment in all tested genes (Figure 2A). Similar results were obtained by isolate ASY 34 (Figure 2B) with the *Cel*, *Peh*, *Pel* and *Xy* genes, but the *Prt* gene behaved differently since the expression of this gene was higher under the control conditions than the induced conditions. The high expression level of the *Prt* gene in isolate ASY 34 under the controlled conditions needs further investigation for explanation. With these findings this study could confirm the hypothesis that the presence of potatoes tissues stimulates the expression of pathogenicity genes in both high disease severity (isolate ASY 7) and weak disease severity (isolate ASY 34).

A comparison of the expression fold change of the five genes between isolate ASY 7 and isolate ASY 34 were made under both controlled (Figure 3A) and induced conditions (Figure 3B).

Under the controlled conditions (without adding the potato tissues to the bacterial cultures), all the genes found to be expressed in the two isolates although the absence of the potato's tissues in the cultures (Figure 3A). This means that the genes were expressed continuously without being hosted on the potato's tubers but with low expression levels, indicating a potential early role of these genes in the pathogenicity towards the infection of potatoes tubers by soft rot bacteria.

Surprisingly, the data in Figure (3A) shows that isolate ASY 34 expressed the five gene higher than isolate ASY 7 under the controlled conditions (when no potato tissues were added to the bacterial cultures). These data contradict with the disease's severity data (Table 2) since isolate ASY 7 caused higher diseases severity compared to isolate ASY 34. These results means that the genes are expressed continuously and the amount of expression before the infection are not a good indicator for the diseases severity. On the other hand, each strain expressed differently diseases severity during and after infecting the potatoes tubers.

Since isolate ASY 7 caused higher diseases severity and was more aggressive than isolate ASY 34 in infecting the potato tubers (Table 2), so it was expected that isolate ASY 7 will more highly express all the pathogenic genes under the induced conditions (when the potato tissues were added to the

bacterial cultures) than isolate ASY 34. But the qRT-PCR results under the induced conditions (Figure 3B), reveled a higher expression levels of only *Peh*, *Prt* and *Xy* genes in isolate ASY 7 when compared to isolate ASY 34 (these genes agree with the disease severity results). On the other hand, the expression of the *Cel* and *Pel* genes were higher in isolate ASY 34 when compared to isolate ASY 7 (these genes results contradict with the disease severity results). This means that *Peh*, *Prt* and *Xy* genes are playing a higher role than *Cel* and *Pel* genes in the infection and development of soft rot disease process in potato's tubers.

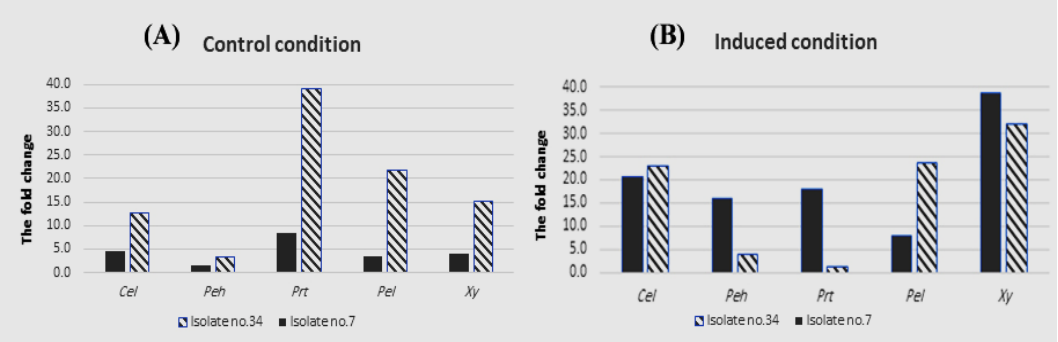

Figure 3. The fold change in the expression of the different genes showing a comparison between isolate ASY 7 and isolate ASY 34 under both (A) the controlled conditions and (B) induced conditions.

To sum up, our findings confirmed that the tested pathogenicity genes are expressing continuously by *P. carotovorum* without being hosted on the potato's tubers but with low levels indicating that these genes are potential for the initiation of the infection. But the amount of expression before the infection are not correlated with the final disease severity which means the presence of other factors affecting disease severity.

After the infection, these genes are highly expressed which means they are stimulated by the potato tissues. In addition, the *Peh*, *Prt*, and *Xy* genes are more important and playing higher role than the *Cel*, and *Pel* genes after the infection process of the potato's tubers. (Oeser *et al*. (2002) found that Polygalacturonase (*Peh*) expression is necessary for a successful infection between *Claviceps purpurea* and rye. Also, Zhang *et al.* (2005) studies on *Rhizoctonia solani* demonstrated that polygalacturonase production caused membrane damage, chlorosis, and tissue maceration in rice sheath tissue.

Charkowski *et al*. (2012) stated that *Pectobacterium* spp. major virulence factor are the secretion of the plant cell walls degrading enzymes; specifically, pectinases, hemicellulases and proteases. The results obtained in this study under the induced condition (Figure 3B) showed that the expression of the cellulase gene was almost the same in both weak (ASY 34) and strong (ASY 7) tested isolates. Which this result concluded that cellulase is important factor for the infection, but it is not the major factor for diseases severity.

It seems that the regulation of the plant cell wall–degrading enzyme genes (PCWDEs) in *P. carotovorum* are very complicated. Many of these genes were the master regulator of virulence, although many studies were performed to identify the factors controlling their expression (Burr *et al*., 2006; Sjöblom *et al.*, 2006 and Deng *et. al.*, 2014).

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تقدير التعبير الجيني للجينات الممرضة في بكتريا بكتوباكتيريم كاروتوفورام باستخدام تفاعل البلمرة المسلسل الكمي

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يعد مرض العفن الطري البكتيري من أكثر الأمراض البكتيرية أهمية واكثرها انتشارًا والتي تتسبب في خسائر فادحة ومدمرة للمحصول مما يؤدي إلى فقدان مئات الأطنان في غضون أيام. يصيب مرض العفن الطري درنات البطاطس بالإضافة إلى العديد من محاصيل الخضروات الأخرى. في هذه الدراسة، تم عزل أربع عزلات مختلفة من بكتيريا *Pectobacterium carotovorum* المسبب الرئيسي لمرض مرض العفن الطري البكتيري من درنات بطاطس متعفنة طبيعياً، وتم إخضاع العزلات لاختبار القدرة المرضية لدرنات البطاطس وأظهرت النتائج أن العزلات اختلفت من حيث قدرتها علي الاصابة وشدة الاصابة. كما تم استخدام تقنية تفاعل البلمرة المتسلسل الكمي (qRT-PCR) للتحقق من تأثير وجود أنسجة البطاطس على تحفيز الجينات المسببة للمرض. تمت دراسة تعبير خمسة جينات مسببة لمرض العفن الطري هي: polygalacturonase (Peh)، pectate lyase (Pel)، cellulase (Cel)، xylanase (Xy) و protease (Prt). أكدت النتائج أن وجود أنسجة البطاطس يحفز التعبير الجيني لهذه الجينات المسببة للمرض في كلا من العزلات ذات القدرة المرضية الشديدة والضعيفة.