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Differentiation between *Ralstonia solanacearum* Isolates Based on Polygalacturonase (PEHA) Gene, Protein Profile and PEHA Gene Expression

Sawsan S. EL-Shamy *

Division of plant pathology, Faculty of Agriculture, Damanhur University, El-Beheira, Egypt,

ABSTRACT

Ralstonia solanacearum is the bacterium that causes brown rot in potatoes. It poses a serious threat to the global output of potatoes, especially in warm-growing regions like the Mediterranean. Infections with brown rot can be extremely harmful and result in significant yield losses. Several extracellular enzymes that degrade plant cells wall are produced by *Ralstonia solanacearum* and are considered virulence factors. These comprise two exopolygalacturonase -PGs, pehB and pehC, and one endopolygalacturonase (PG), pehA. Five isolates of *R. solanacearum* were isolated and identified, namely BR1, BR2, BR3, BR4, and BR5. After the isolates' pathogenicity was assessed, it was found that isolates BR3 and BR4 were extremely virulent, isolate BR2 was moderately virulent, and the virulence of isolates BR1 and BR5 was weak mildly. To investigate the existence of the Polygalacturonase (PehA) gene, a specific primer was employed in *R. solancearum* isolates to identify molecular weight 500 bp present in all isolates. Every isolate has a band with a molecular weight of roughly 52 kilodaltons (kDa) visible on the SDS/PAGE gel. The highest of the gene expression level of pehA gene was recorded in NB+PGA than NB+potato tubers compared with the gene expression level in NB medium (control). Moreover, the gene expression level was high in BR3 isolate expression and BR4 isolates, while the low gene expression was observed at BR1 and BR5 isolates. The BR2 isolate was moderate gene expression.

Keywords: Potato, enzymes, Polygalacturonase, pathogenicity, gene expression

INTRODUCTION

The gram-negative, soil-borne bacterium *Ralstonia solanacearum* can cause wilting in over 200 plant species across 50 families (Hayward 2000; Schell 2000). Bananas, tobacco, potatoes, geraniums, and tomatoes are some of its primary hosts. *R. solanacearum* consistently results in significant output loss both for business and survival cultivation worldwide. *R. solanacearum* often spreads throughout the vascular system after entering the xylem through wounds or the locations where secondary roots emerge on plants. Dysfunctional vascular function by extensive colonization, causes the plants to wilt and perish quickly. Nonwooy stem cells are not visibly macerated until the disease is advanced.

Numerous investigations have evaluated the variation in the genetic makeup of *R. solanacearum* strains acquired from various host species and geographical locations (Cook et al., 1989). Research on the genetic and phenotypic variance among populations of *R. solanacearum* in certain geographic areas has also been conducted. (Dookun *et al.,* 2001; Horita and Tsuchiya 2001). There is a growing global number of infected sites, which highlights the requirement for effective identification tests in epidemiological research and material exchanges (Castillo and Greenberg, 2007). Polymerase chain reaction (PCR) is the recognized and suggested method for *R. solanacearum*, identify (Kutin *et al.,* 2009). Various sections of the *R. solanacearum* genome (Taghavi *et al.,* 1996). These comprise ribosomal genes, specifically the sequences of 16S rDNA (Seal *et al.*, 1993) and between 16S and 23S, the internally transcribed spacer region (Pastrik *et al.,* 2002). The region of the hrp gene (Poussier and Luisetti, 2000), the polygalacturonase gene (Gillings *et al.,* 1993), and insertion sequences (Lee *et al.,* 2001).

The virulence factors of *R. solanacearum* are quantitative, contributing varying degrees of pathogen aggression. Numerous recognized virulence factors, such as endoglucanase, three PGs, and extracellular polysaccharide (EPS I), are secreted (Huang and Allen (1997), Denny and Baek (1991), Roberts et al. (1988), Schell et al. (1988). It appears that a group of three PGs that break down plant cell walls helps in *R. solanacearum's* plant invasion. The three PGs have different reaction products even though they all hydrolytically break the pectic polymer apart. PehA, also called PglA, is an endo-PG that randomly breaks the pectic polymer apart. inside, releasing big oligomers, mostly trimers of galacturonic acid (Roberts *et al.,* 1988 and Allen *et al.,* 1991). At the nonreducing end of pectin, the pectic polymer is broken by PehB and PehC, an exo-Polygalacturonase releasing the galUA dimers and monomers (Huang and Allen 1997). pH 5.5 was the ideal value for polygalacturonase activity (Ofuya 1984).

Following the enrichment of the *R. solanacearum* population through growth on SMSA, 10 to 30 cfu/ml (colony forming units) of extract from potato tubers can be detected using real-time PCR with TaqMan fluorescent probes (semiselective medium from South Africa) (Weller *et al.,* 2000 and Ozakman and Schaad, 2003). Additionally, this technique removes the need for post-PCR analyses, yields the target sequence's quantitative estimate in a sample and shows that *R. solanacearum* cells are viable when the target sequence content rises in samples that are periodically taken out of the enrichment culture. The aim of the present study is isolation, purification and *R. solanacearum* isolates

* **Corresponding author.**
E-mail address: Sawsan.elshamy@agr.dmu.edu.eg
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identification was gathered from fields, study the pathogenicity test to determinene the difference in of virulence between the isolates, Bacterial identification using PehA gene amplification and PehA gene expression quantification using real-time PCR.

MATERIALS AND METHODS

1.Sampling

Within the Governorate of El-Behiera, potato-infected samples were gathered from marketplaces, cold storage facilities, and fields. They were then transported to the laboratory in separate polyethylene bags. The names of the isolates were BR1, BR2, BR3, BR4, and BR5.

2. The causal organmism isolation and purification

Tetrazolium agar medium (TZC) was streaked with a loopful of bacterial suspension after oozes and vascular tissues of infected potato tubers that are discolored were submerged in sterile water (Abo-El-Dahab and El Goorani, 1969). Agar 15 g, glycerol 20 ml, peptone 5 g, beef extract 3 g, distilled water 1, and 0.05% TZC (pH 7.0) are the ingredients. For 48 hours, the plates were incubated at 28 ºC. The TZC medium was used to differentiate fluidal colonies of virulent isolates (Kelman, 1954). *Ralstonia solanacearum* strains, both virulent and avirulent, were routinely grown on glycerol nutrient agar (GNA) medium at room temperature.

3. Pathogenicity test:

We tested the pathogenicity of *Ralstonia solanacearum* isolates on the highly susceptible Spunta potato cultivar. The previously mentioned cultivar potato tubers were sterilised on their surfaces for five minutes using 1% sodium hypochlorite, then they were cleaned with sterile water and placed in 15 cm diameter plastic pots that were filled with sterile peat moss and clay (one tuber per pot).

After plants grew to a height of 15-20 cm, a sterilized needle was used to pierce 0.25 ml of suspension of bacteria (10^9 cfu/ml; A600, 0.5) and inject it into the stems five cm above the ground (Prior and Steva, 1990). The isolates were used to inoculate plants, which were kept at 25 + 2ºC in a greenhouse. In four replicates, plants that were injected with sterile distilled water acted as the control. Every day, the degree of wilting was noted using the He *et al.* (1983) scale as follows:- One = absence of symptoms, Two indicates one wilted leave, Three indicates two or three wilted leaves, four indicates four or more wilted leaves, and five indicates a dead plant, the readings were taken up to 5 weeks after inoculation. The disease's severity was determined 19 days after inoculation (dpi). Using the formulas provided by Bereika *et al.* (2020), the disease severity percentage (DS%) was computed as follows: $(ni \times vi) / N \times S\} \times 100 = DS\%$ Where N is the total number of plants observed, Σ = Summation; ni shows how many plants are included in each disease rating., vi represents the disease rating, S symbolizes the highest illness rating, and N symbolizes the total number of plants that have been observed.

4. Bacterial identification using PehA gene amplification:

1. Extraction of genomic DNA from *R. solanacearum* isolates.

Using the wizard genomic DNA purification kit QIAGENE DNA purification kit (Germany) under manufacturing protocols, the genomic DNA of the bacteria was extracted.

2. Amplification of PehA gene

Using specific primer of PehA gene forward, 5`CAAGTCGGGCGTGACGCTGT`3and Reverse, 5` GACCTTGTCGGGCGTGGAGC`3 were used to amplify approximately the 500bp of the PehA gene. The PCR reaction was performed on the genomic DNA under the following conditions: 94 ºC for 5 minutes was the initial cycle, and 34 cycles of 95 ºC for 1 minute, 60 ºC for 1 minute, and 72 for 1 minute were performed, followed by an extension cycle at 72 for 10 minutes. The PCR result was seen on 1% agarose gel, and a gel documentation system was used to take pictures.

5. Polyacrylamide gel electrophoresis using sodium dodecylsulfate (SDS-PAGE)

- Preparation of sample for SDS-PAGE

The *Ralstonia Solanacearium* The isolates were cultured for 48 hours at 30 ºC ±2 in shake flasks at 150 rpm of nutrient Broth (NB) medium (the sole source carbon enhanced with 0.2% polygalacturonic acid). Centrifugation was used to harvest the additional cells for 10 minutes at 10,000 rpm, and the supernatant was disposed of. A portion of the entire cell pellet was preserved and kept at -8ºC in tube. Reusing a large probe with 9.0 second bursts that are separated by one minute, the remaining pellet was sonicated in an ice bath using lysis buffer (pH 8.8, Tris HCl 50 mM, 2-BMES mM, and PMSF 1 mM). Samples that had been sonicated were centrifuged for ten minutes at 10,000 rpm, and the supernatant was transferred into a centrifuge tube. These were centrifuged for 90 minutes at 30,000 rpm, and the membrane pellets that were produced were kept at -20°C. The SDS-PAGE analysis of the sample was conducted according to Piñeiro *et al.* (1999).

6.Polygalacturonase (pehA) gene expression quantification via real-time PCR

1. Samples preparation

For 48 hours at 30 degrees Celsius ±2, five isolates were cultured in NB medium with 0.2% polygalacturonic acid (PGA) added as a supplement as the only source of carbon and Isolation of RNA. These isolates were grown in NB medium without polygalacturonic acid (PGA) being used as controls. The isolates were grown in NB medium added to it peeled potato periderm and cortex tissue (can be cut potato tuber in a blender) for 48 h at 30 ºC ±2 and Isolation of RNA.

2. Isolation of total RNA from (NB medium+ PGA), (NB medium + potato tuber) and NB medium (control)

Total RNA was isolated from (NB medium+ PGA), (NB medium + potato tuber) and NB medium (control) utilizing the Guanidium Thiocynate Method with the GStractTM RNA Isolation kit.

3. Reverse Transcription Polymerase Chain Reaction (RT- PCR) Analysis:

When reverse transcriptase and dNTPS were present, reverse transcription (RT), referred to as the initial strand reaction, served as the means of converting mRNA into complementary DNA (cDNA). The parts are mixed for an hour at 42°C with a DNA primer in a reverse transcriptase buffer.

Reverse transcription of polymerase chain reaction exponential amplification offers a very sensitive method that can detect RNA molecules at very low copy numbers.

The primer 5'-TTTTTTTTTTTTTTT-3' was used for the oligo(dT) reverse transcription reaction. 2.5 µl (5x) buffer containing MgCl2, 2.5 µl (2.5 mM) dNTPs, 1 µl (10 pmol) primer, 2.5 µl RNA (2 mg/ml), and 0.5 unit reverse transcriptase enzyme were included in each 25 µl reaction

mixture. PCR amplification was carried out in a thermal cycler set for one hour at 42 °C.4.

4. Using real-time PCR to quantify the expression of the pehA gene

Each reaction tube for samples analyzed with the Fermentase kit held 12.5 µl of 2x Quantitech SYBR® Green RT Mix, 1 µl of each of the forward and reverse primers (25 pm/µl), and 9.25 µl of RNase-free water, for a total of 25 µl.

Samples were spun before loading in the Rotor's wells. Template cDNA ≤500 ng, the sequence of polygalacturonase (pehA) primer were used

Forward primer: 5' CAAGTCGGGCGTGACGCTGT 3'

Reverse primer: 5' GACCTTGTCGGGCGTGGAGC 3'

According to Sambrock and Russell (2001), the forward and reverse 16S rRNA primers were 5AGG ACG TGC TCC AAC CGC A ~3 and 5AAC TGG AGG AAG GTG GGG AT ~3. The real-time PCR program was as follows : initial denaturation at 95 °C for 10 min.; 40 cycles of 95°C for 15 sec.; annealing at 60°C for 30 sec and extension at 72 °C for 30 sec. Data collection was done in the extension phase. Utilizing the Rotor-Gene6000system (Qiagen, USA), this reaction was carried out.

5. Real-time PCR data analysis

Utilizing a Rotor-Gene 6000 (Qiagen, ABI System, USA), the reaction was carried out. According to Livak and Schmittgen (2001), ΔCq=Cq–reference gene, ΔΔCq=ΔCq–control, and ΔΔ C q expression = $2^{(-\Delta\Delta Cq)}$ were used to perform relative quantification of gene expression. Every time, the untreated control plants' relative expression was set to 1., and the target genes' expression levels were normalized with the 16S rRNA gene

To estimate the relative expression of genes using real-time PCR, Real-time PCR datasets for the sample and control were examined using the proper bioinformatics and statistical programs. The results were then normalized to the 16S rRNA housekeeping gene (Reference gene). Rotor-Gene-6000 version 1.7 was used to statistically evaluate, interpret, and analyze the data.

RESULTS AND DISCUSSION

Results

Isolation and identification of bacterial isolates experiments

From diseased potato tubers, five isolates of *R. solanacearum* bacteria were obtained on tetrazolium agar medium (TZC) BR1, BR2, BR3, BR4 and BR5. It is clear from the obtained data of the isolates in figure (1) that the obtained five isolates were identified as *R. solanacearum.*

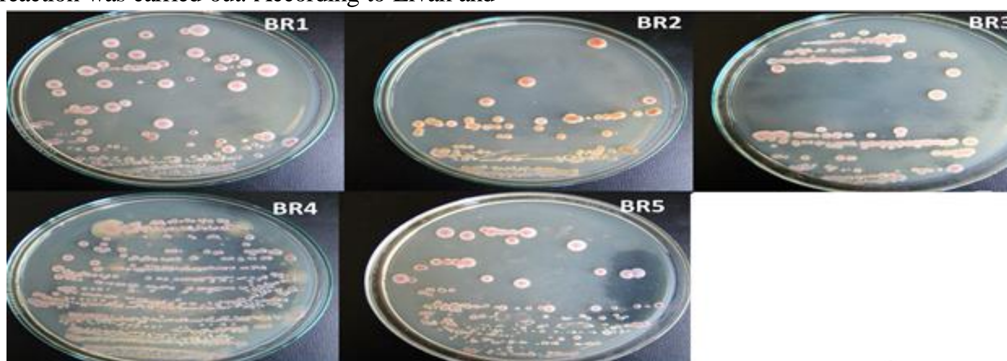

Fig. 1. Five isolates of *R. solanacearum* bacteria were isolated on tetrazolium agar medium (TZC)

Pathogenicity test

The result of the Pathogenicity test was published in the first paper of this study (Eid and Shamy, 2021). The potato cultivar of Spunta, which is known to be extremely susceptible to infection by *R. solanacearum*, was used to test the pathogenicity of the five isolates of *R. solanacearum*. All of the isolates were virulent, according to the data in Fig. (2) and Table (1); however, according to He *et al.* (1983), BR3 and BR4 isolates were extremely virulent, with no significant difference between them, while the isolates BR2 were moderately virulent and BR1 and BR5 isolates were weakly.

Fig. 2. Nineteen days following inoculation, pathogenicity tests were carried out on the Spunta cultivar utilizing five isolates of *R. solanacearum* (BR1, BR2, BR3, BR4, and BR5).

Table 1. Pathological response of the Spunta cultivar to five isolates of *R. solanacearum*

BR1	24 ± 0.056^{c}
BR2	42 ± 0.156^{b}
BR3	73 ± 0.090^{a}
BR4	70 ± 0.065^{a}
BR5	20 ± 0.166^{c}

*** Data were an average of four replicates. LSD0.05 for BR isolates = 5.017**

Identification bacterial isolates by Polygalacturonase (PehA) gene

Using a specific primer, the presence of the Polygalacturonase (PehA) gene in five isolates of *R. solanacearum* was investigated. Fig (3) showed that there are one band at molecular weight 500 bp present in all isolates. These results confirm that the isolates are *R. solanacearum.*

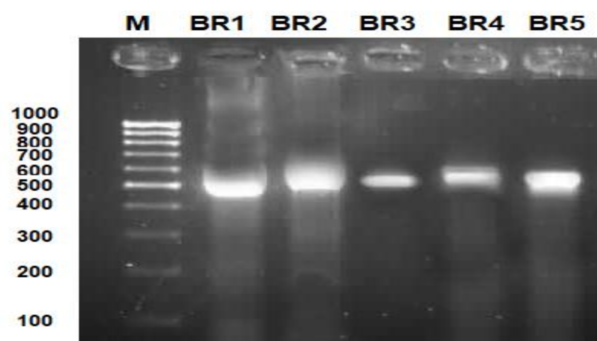

Fig. 3. Agarose gel electrophoresis of polygalacturonase (pehA) PCR product (500 bp), M: DNA marker. Lanes BR1, BR2, BR3, BR4 and BR5 are *R. solanacearum* isolates

Protein profile of bacterial isolates by SDS-PAGE

SDS/PAGE analysis was performed on all isolates were grown on NB medium added 2 % polygalacturonic acid (PGA) and secreted the PG. This protein should be expressed in these conditions. Purification and characterization of PG. polygalacturonas (PG) was purified from the culture supernatant of *R. solanacearum* isolates as described in Materials and Methods. The molecular weights of PG about 52 kilodaltons (kDa). The SDS/PAGE gel Figure (4) shows a band that is present in all isolates within this molecular weight range.

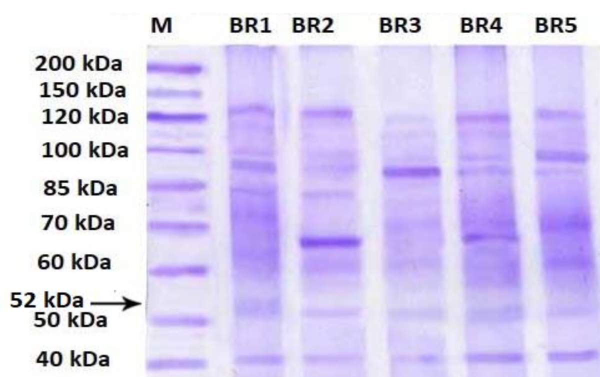

Fig. 4. SDS-PAGE analysis of polygalacturonaes (PG): M) protein molecular weight standard, (BR1, BR2, BR3, BR4 and BR5) *R.solanacearum* isolates. The methods outlined in Materials and Methods were followed to prepare the protein samples. Following a 5-minute heating period at 100°C, each sample was combined with loading buffer and loaded onto a 5.9% SDS-polyacrylamide gel. Coomassie blue staining was applied to the gel.

Polygalacturonase (pehA) gene expression quantification via real-time PCR

The result in fig (5) and table 2 showed the mRNA relative amounts detected from *pehA* gene present in NB medium+ PGA, NB medium + potato tuber and NB as a control were obtained after inoculation with *R. solanacearum* isolates were compared with the amount of mRNA that in control (NB medium) and the results normalized to 16s gene (reference gene or housekeeping gene). Where the highest level of gene expression of *pehA* gene was recorded in NB+PGA than NB+potato tubers compared with the level of gene expression in NB medium (control). Moreover, the level of gene expression was high at BR3 isolate expression and BR4 isolates, while the low gene expression was observed at BR1 and BR5 isolates. The BR5 isolate had moderate gene expression.

Table 2. Effect of NB media, NB+PGA media and NB+potato tuber media on quantitative induction of pehA gene expression.

	BR1	BR2	BR3	BR4	BR5	Mean
NB(Control)	0.96	0.62	0.7	0.65	0.57	0.70^{c}
NB+PGA	1.45	2.00	2.54	2.32	1.32	1.96^{a}
NB+Potato tuber	1.1	1.87	2	1.92	0.98	1.57^{b}
Mean	1.17^{c}	1.50^{b}	1.75^{a}	1.63^{a}	0.96^{d}	

*** Data are average of three replicates.**
$LSD_{0.05}$ for bacterial isolates = 0.12
$LSD_{0.05}$ for bacterial media = 0.1

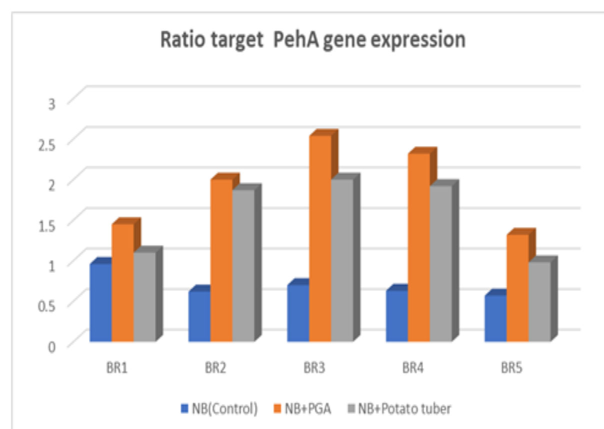

Fig. 5. Effect of NB media, NB+PGA media and NB+potato tuber media on quantitative induction of pehA gene expression.

Discussion

In order to mitigate the significant losses that *R. solanacearum* causes in tropical and subtropical regions, the most critical aspect of disease management is prompt pathogen identification. As far as is known, no precise and sensitive real-time PCR test is available for directly identifying *R. solanacearum* in soil and plant samples devoid of phage and culture, despite the publication of numerous related techniques and marker genes. Citations: Poussier *et al.* (2000), Thammakijjawat *et al.* (2006), Kutin *et al.* (2009), Kang et al. (2007). In the current study five *R. solanacearum* isolates bacteria were purified from infected tubers. The data obtained that all seventy isolates could be identified as *R. solanacearum.* This result agreed with the results obtained by (Fahy and Persley, 1983 and Adhikari, 1993). Pathogencity test results showed varying degrees of wilting caused by the infection with *R. solanacearum* isolates. The isolates BR3 and BR4 were more aggressive no significant different was found between them, while moderate virulence was observed in the BR2 isolate and weak virulence was observed in the isolates BR1 and BR5.

Other studies have reported that the virulence of *R. solanacearum* isolates varies amongst potato cultivars (El-Ariqi, 2008, EL-Gayar, 2003; Khairy *et al.,* 2021) Polygalacturonase gene (PehA) was used to study the identification of the isolates and study the genetic variability between the five isolates of *R. solanacearum.* In our investigation PehA gene (500 bp) was used to in *R. solanacearum* isolates detection; all the isolates gave one

band in the right expected molecular weight. The obtained DNA sequences revealed that the sequences belong to *R. solanacearum*. Such data agreed with the finding of Adss (2014) and Van der Wolf *et al.,* (2004). In this study the molecular weights of PG are about 52 kilodaltons (kDa). The *R. solanacearum* CWDE's involvement has been investigated in strains AW and K60, typically with tomato or eggplant as the host. The SDS/PAGE gel Figure demonstrates the existence of a band in this range of molecular weights found in all isolates (González and Allen (2003), Huang and Allen (1997), Denny et al. (1990). Five exoenzymes are produced by these extremely similar strains: one endopolygalacturonase (PehA or PglA), Two exopolygalacturonases (PehB and PehC), one β-1,4-endoglucanase (Egl), and one pectin methyl esterase (Pme). It is unknown if *R. solanacearum* produces any pectate lyase (Pel) enzymes. Genetic inactivation of individual genes has demonstrated that the wild-type's capacity to induce wilt is largely dependent on Egl, PehA, and PehB, with PehB being the least significant of the five exoenzymes (Huang and Allen (2000); Denny et al., 1990). In this study, the genes that were found to be differentially expressed in the bacterial isolates were grown in NB medium that was supplemented polygalacturonic acid 0.2%. Peeled potato periderm and cortex tissue were added to the NB medium, which was used as a control. And might therefore encourage differentiating gene expression. Pectin, a component of plant cell walls, is known to be broken down by a variety of enzymes during plant infection by *Ralstonia solanacearum*. NB is a standard laboratory complete medium. The highe level of gene expression of pehA gene was recorded in NB+PGA than NB+potato tubers compared with the level of pehA gene expression in NB medium (control). Moreover, the level of pehA gene expression was high at BR3 isolate expression and BR4 isolates, while low gene expression was observed at BR1 and BR5 isolates. The BR5 isolate had moderate gene expression. These results agreed with the data obtained from the pathogenicity test. PehA, PehB, and PehC, three plant cell wall-degrading PGs, have been identified as virulence factors that promote *R. solanacearum* plant invasion. The three PGs have different reaction products even though they all hydrolytically cleave the pectic polymer. According to Allen *et al.* (1991) and Roberts *et al.* (1988), PehA, an endo-PG , randomly Internal cleavage of the pectic polymer, the release of big oligomers, primarily trimers of galacturonic acid (galUA). At the pectin's nonreducing end, the exo-PG PehC and exo-poly-D-galacturonosidase PehB cleave the pectic polymer to release the galUA dimers and monomers, respectively (Huang and Allen 1997).

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التفريق بين عزلات بكتريا *Ralstonia solanacearum* على أساس جين البولي جلاكتويورينيز (PEHA) والبروتين والتعبير الجيني لجين PEHA

سوسن صلاح الشامي

قسم أمراض النبات , كلية الزراعة, جامعة دمنهور, البحيرة, مصر

الملخص

تتسبب بكتيريا *Ralstonia solanacearum* في مرض العفن البني في البطاطس. تنتج *Ralstonia solanacearum* العديد من الإنزيمات المحللة لجدار الخلايا النباتية خارج الخلية. ومنها انزيم واحد من النوع endopolygalacturonase (PG): pehA ، واثنين من نوع exo-PGs:pehB and pehC. تم دراسة خمس عزلات من *R. solanacearum* (BR1، BR2، BR3، BR4، وBR5). تم عزلها وتحديد هويتها. تم تحديد القدرة المرضية للعزلات، وأظهرت النتائج أن العزلتين BR3 و BR4 كانتا شديدتي القدرة المرضية، في حين كانت العزلة BR2 متوسطة القدرة المرضية والعزلتين BR1 و BR5 كانتا ضعيفتي القدرة المرضية. تم استخدام بادئ متخصص لدراسة وجود جين Polygalacturonase (PehA) في عزلات *R. solancearum*. بستخدام تقنية SDS/PAGE وجد باند عند الوزن الجزيئي 52 (kDa) في جميع العزلات. تم تسجيل أعلى مستوى للتعبير الجيني لجين pehA في NB+PGA مقارنة مع درنات البطاطس NB+ مقارنة بمستوى التعبير الجيني في بيئة NB (كنترول). علاوة على ذلك، كان مستوى التعبير الجيني مرتفعا في العزلات BR3 و BR4، في حين لوحظ انخفاض التعبير الجيني في العزلات BR1 و BR5. كانت عزلة BR2 متوسطة التعبير الجيني.

الكلمات الدالة: انزيم ، البطاطس، القدرة المرضية, التعبير الجيني