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RESEARCH

# Manuka Honey Reduces the Virulence of *Pectobacterium brasiliense* by Suppressing Genes That Encode Plant Cell Wall-Degrading Enzymes

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ABSTRACT The primary virulence of soft rot pectobacteria, including *Pectobacterium brasiliense*, is mainly determined by the massive production of plant cell wall-degrading enzymes (PCWDEs), which promote plant tissue maceration in many crops. The antibiotic treatment to treat bacterial soft rot causes environmental problems and potentially affects resistance. Antibiotic resistance is driving interest in antimicrobial treatments, and no organism has been reported to have acquired resistance to honey. However, the use of honey as a therapeutic agent for plant bacterial pathogens has rarely been studied. Therefore, this study was undertaken to determine the *in vitro* effect of Manuka honey against *P. brasiliense* at the phenotypic and genotypic levels. A sublethal concentration of honey was determined by a growth inhibition assay in broth medium containing different concentrations of Manuka honey. A macerating assay was performed on orchid leaves, and the activities of the PCWDEs were examined in plate assays. The expression of PCWDE-associated genes was investigated using semi-quantitative reverse transcription-polymerase chain reaction analysis. The exposure of *P. brasiliense* to a sublethal concentration of Manuka honey significantly decreased the maceration ability of the orchid and the synthesis of PCWDEs, i.e., pectate lyase, polygalacturonase, and protease. Moreover, five PCWDEs-encoding genes, such as *pelA*, *pelB*, *pelC*, *pehA*, and *prtW*, had lower expression levels after the honey treatment compared with *recA* as the internal standard. The honey treatment decreased the virulence of *P. brasiliense* associated with the synthesis of PCWDEs. Therefore, Manuka honey reduced virulence by suppressing the expression of the PCWDE genes.



## 1. INTRODUCTION

Bacterial soft rot is a complex disease and a major agricultural concern. This disease is caused by bacteria from the genera *Dickeya* and *Pectobacterium*, which are also the most studied soft rot pathogens (Charkowski 2018). Both genera have broad host ranges due to their ability to infect approximately 35% of angiosperm species (Ma et al. 2007). Bacteria causing soft rot and blackleg produce pectinolytic enzymes, which kill or rot plants during storage (Silva et al. 2020). Bacterial pathogens rely on quorum sensing (QS) to coordinate the virulence factors that produce plant cell wall-degrading enzymes (PCWDEs) (Joshi et al. 2016). PCWDEs, the hrp and hrc genes, the type of secretion system, and carotovicin affect the pathogenicity of *Pectobacterium*. The PCWDEs related to soft rot include polygalacturonase (Peh), pectate lyase (Pel), cellulase, xylanase, and protease (Prt) (Lee et al. 2014). Additionally, soft rot bacteria also respond to small molecules in their environment, such as sugars released by plants, after plant cell wall degradation and metabolism of pectate (Joko et al. 2018).

*Pectobacterium brasiliense* is a member of the soft rot *Enterobacteriaceae* family; it possesses a very high virulence and causes blackleg (Kwenda et al. 2016) and soft rot disease in a wide range of hosts. The current management of this disease relies on synthetic pesticides and antibiotics; however, the extensive use of these substances increases the risk that bacterial populations will develop resistance; thus, threatening human and environmental health (Popović et al. 2017).

Antibiotics have been used to manage several plant pathogenic bacteria on high-value crops to avoid severe economic losses. However, the presence of plant pathogenic bacteria that are resistant to antibiotics hinders management, and antibiotic resistance by such pathogens has been reported to evolve through horizontal gene transfer (Sundin and Wang 2018). Honey has antibacterial properties and has been used to manage antibiotic resistance (Carter et al. 2016). Manuka honey contains natural compounds reported to have antimicrobial and or antivirulence activities, such as methylglyoxal (MGO), and leptosperin (Roberts et al. 2015). Manuka honey affects the growth of several bacteria, such as *Streptococcus pyogenes* (Maddocks et al. 2012), *Pseudomonas aeruginosa* (Okhiria et al. 2009), and *Staphylococcus aureus* (Almasaudi et al. 2017). This study aimed to determine the effects of Manuka honey on

*P. brasiliense* virulence, such as the production of PCWDEs and the quantitative expression of PCWDE-encoding genes and their virulence on host plants.

## 2. MATERIALS AND METHODS

### 2.1 Bacterial growth conditions

The *P. brasiliense* used in this study was grown on yeast peptone agar (YPA) medium (0.5% yeast extract; 1% peptone; 1.5% agar). The bacterial cultures were incubated for 1–2 days at room temperature (±27°C), and single colonies were regularly cultivated in new media to ensure bacterial viability and the purity of the isolates (Fauzia and Joko 2021). An overnight culture of *P. brasiliense* on YPA was converted into a suspension culture with an OD600 nm value of 0.2 (108 CFU) and then grown on 5 mL of yeast peptone broth (YPB) medium. The Manuka honey originated from monofloral Manuka (*Leptospermum scoparium*) trees in New Zealand and was used as the treatment in this study. The Manuka honey possessed a unique Manuka factor value (UMF) of 20+ (Streamland) (contained 829 ppm MGO, 200 ppm leptosperin, and 77% sugar). The Manuka honey was prepared at concentrations of 10% (w/v), 5% (w/v), and an untreated control (0% w/v). Cell density was measured every 2 h using a Genesys 10S UV-VIS spectrophotometer (Thermo Scientific, Waltham, MA, USA).

### 2.2 Pathogenicity assay

The pathogenicity assay was performed on an orchid (*Dendrobium burana* cv. Emerald). The orchid leaves were mechanically injured using a syringe. A 10 µL bacterial suspension grown on YPB, containing 5% or 0% (untreated control) Manuka honey, was used to infect wounds using a micropipette. The orchid plants were later bagged, and wet cotton was inserted into the bags to maintain the humidity.

### 2.3 Enzyme assays

The activities of the extracellular enzymes were tested to identify the effects of Manuka honey on bacterial enzyme production. We prepared a medium consisting of 1% (w/v) polygalacturonic acid, 1% (w/v) yeast extract, 0.38 µM CaCl2, 100 mM Tris-Cl (pH 8.5), and 0.8% (w/v) agarose to test the production of Pel. Peh enzyme production was tested on medium containing 1% (w/v) polygalacturonic acid (w/v), 1% (w/v) yeast extract, 2.2 mM ethylenediaminetetraacetic acid, 110 mM sodium acetate (pH 5.5), and 0.8% (w/v) agarose. The media were plated in Petri dishes, with two wells of each medium used for the settled media, and 15 µL liquid agarose was placed in these wells and allowed to dry. A total of 30 µL of the bacterial supernatant was added to each well. The right side of the bacterial supernatant was treated with Manuka honey 5% (w/v), whereas the left side contained the untreated bacterial supernatant. The Petri dishes were incubated for 16–18 h. HCl (4 N) was placed on the top of the media for the Pel and Peh tests, and transparent zones were observed. Bacterial gelatin liquefaction activity was tested to determine the effect of the Manuka honey on bacterial proteolytic activity in dissolving gelatin. The gelatin consisted of 3 g beef extract, 5 g peptone, 120 g gelatin, and 1 L distilled water. Ten µL of 24-h-old P. brasiliense isolates were inoculated into the medium using the stab method. The bacteria were incubated at room temperature and observed by placing the medium at 4°C for 30 min to observe dissolution.

### 2.4 Selection and primer design of genes involved in virulences

The virulence genes selected in this study were genes that encode the extracellular enzymes important for plant cell wall degradation. The genes selected included those that encode Peh (*peh*A), Pel (*pel*A, *pel*B, *pel*C), and Prt (*prt*W). *rec*A was used as the internal standard for the expression analysis. The primers were designed based on the nucleotide database from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/), and BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to confirm the gene identities. The primers were designed using the Primer3Plus application (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi) (Joko et al. 2019). Table 1 shows the base sequences of the primers. The annealing temperature of each primer was optimized using a gradient thermal cycler (Biorad T100TM, Hilden, Germany).

### 2.5 RNA isolation

*P. brasiliense* was grown on YPB for 12 h with 5% or 0% Manuka honey. Bacteria were obtained by centrifuging the isolates at 10,000 × g for 1 min, and the RNA was isolated using the GENEzolTM Reagent (Ge-neaid, New Taipei City, Taiwan) following the manufacturer's instructions. The quality and quantity of the RNA were determined using the MaestroNano spectrophotometer (MaestroGen Inc., Hsinchu City, Taiwan) (Widyaningsih et al. 2019).

### 2.6 Expression analysis of virulence genes

The expression analysis of the *P. brasiliense* virulence genes was conducted using a two-step reverse transcription-polymerase chain reaction (RT-PCR) method. Bacterial RNA was synthesized into cDNA using the RT-PCR method with the ReverTra Ace-$\alpha$-® kit (TOYOBO, Shiga, Japan,). Semi-quantitative PCR was performed using the bacterial cDNA extracted from bacterial cells grown in YPB media containing 5% (w/v) and the control without the Manuka honey treatment. The PCR was carried out using GoTaq Mastermix (Promega, Madison, WI, USA) with conventional PCR (Biorad T100TM). The PCR conditions were set at a cycle of initial denaturation at 95°C for 3 min, denaturation at 95°C for 1 min, annealing (Table 1) for 40 s, and extension at 72°C for 40 s (Trianom et al. 2019). The PCR products were run on agarose gel electrophoresis and visualized using a UV transilluminator (Promega). The gel images were quantified using ImageJ software with three replicates (Schneider et al. 2012).

## 3. RESULTS

### 3.1 *Pectobacterium brasiliense* growth rate after Manuka honey treatment

A decrease in the growth rate and cell number of *P. brasiliense* occurred after the 5% (w/v) Manuka honey treatment (Figure 1) compared with the untreated control (0% w/v) during the 20 h observation period. *P. brasiliense* did not grow in the 10% (w/v) Manuka honey treatment. These results demonstrate that the 5% (w/v) Manuka honey treatment had sublethal effects and was used in the following experiments.

TABLE 1. Primer sequences for expression analysis of the virulence genes

| Gene | Protein | Sequence (5'->3') | Annealing (ºC) | Temperature | Annealing (ºC) | Temperature |
|---|---|---|---|---|---|---|
| *pelA* | Pectate lyase I | F:TGCCTGGTTTTTACCTACGC<br>R:ATGCGGATGACTGGAAAAGC | 59 | | 122 | |
| *pelB* | Pectate lyase III | F:TCAGCGCATTTTCGAACCAG<br>R:TGGTTCACGCGTACAACAAC | 64 | | 108 | |
| *pelC* | Pectate lyase II | F:CGGCAAGGTTTTTGTTCACG<br>R:ATGCGGAAGACTGGAAAAGC | 64 | | 123 | |
| *pehA* | Endo-polygalacturonase | F:ACGGGGCATTCTCAAAAGAC<br>R:ACAAGGAAAAGCGGTAAGGC | 64 | | 149 | |
| *prtW* | Metalloprotease | F:TTTCACAAAGCCCTGATCGC<br>R:TGGCAGCCAAGGAAATTGTC | 64 | | 134 | |
| *recA* | Recombinase A | F:TGCGTTTATCGATGCTGAGC<br>R:AGCGCGTTAATGCATCACAG | 64 | | 134 | |

### 3.2 *Pectobacterium brasiliense* pathogenicity on orchids

The results showed that soft rot symptoms were less severe on orchids treated with 5% (w/v) Manuka honey (Figure 2a and 2d) compared with the untreated control (Figure 2b and 2c). The diameters of the soft rot symptoms were smaller than those of the untreated control. The soft rot symptoms observed in this experiment included maceration, watery and soft tissues, and browning.

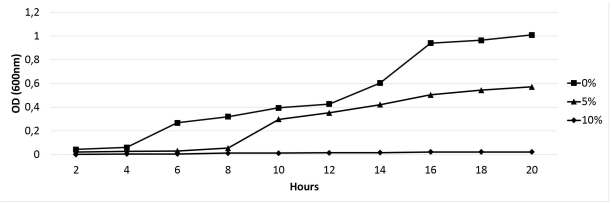


FIGURE 1. Growth rate curve of *Pectobacterium brasiliense* with the 10% (w/v), 5% (w/v), and 0% (w/v) manuka honey treatments. Observations were performed every 2 h using a spectrophotometer (Genesys 10S UV-VIS Thermo Scientific, USA).

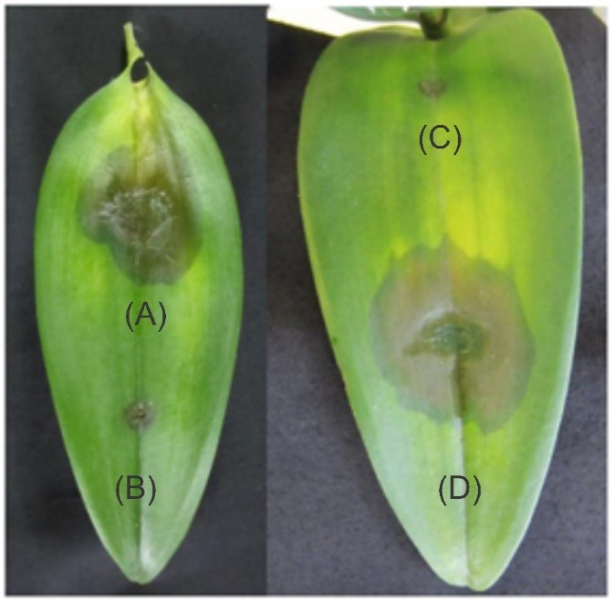


FIGURE 2. *Pectobacterium brasiliense* pathogenicity on orchid (*Dendrobium burana* cv. Emerald) leaves with Manuka honey. (A) and (D) are the untreated control (0% w/v); (B) and (C) are the parts treated with 5% (w/v) Manuka honey.

### 3.3 Extracellular enzymes production by *Pectobacterium brasiliense*

Smaller transparent zones of Pel (Figure 3a) and Peh (Figure 3b) activity were observed in the areas treated with 5% (w/v) Manuka honey compared with the untreated control. These transparent zones suggest that *P. brasiliense* produces extracellular Pel and Peh. The results from the gelatin liquefaction test showed that the gelatin treated with 5% (w/v) Manuka honey and without bacterial inoculation did not dissolve, whereas the gelatin inoculated with bacteria and not treated with Manuka honey dissolved (Figure 3c). These results indicate that Manuka honey prevented the dissolution of the gelatin during the 6-day observation period, revealing proteolytic inhibition.

### 3.4 Detection of virulence genes at the RNA level in *Pectobacterium brasiliense*

The expression of *P. brasiliense* virulence genes was analyzed using two-step RT-PCR, which detected the transcription of low-quantity samples and the number of different genes from the same samples (Sharkey et al. 2004). Figure 4 shows the results of virulence gene testing. The virulence genes detected included *pelA*, *pelB*, *pelC*, *pehA*, and *prtW*. The bacteria treated with 5% (w/v) Manuka honey exhibited different gene expression levels compared with those without the Manuka honey treatment (0%). Gene expression was associated with virulence level. No expression differences in the internal standard were observed (*recA*) between the 5% (w/v) Manuka honey treatment and the untreated control (0% w/v).

### 3.5 Semiquantitative expression analyses of virulence genes in *Pectobacterium brasiliense*

Figure 5 shows that *pehA*, *pelA*, *pelB*, *pelC*, and *prtW* exhibited lower gene expression levels after treatment with 5% (w/v) Manuka honey compared with the untreated control (0% w/v). The expression levels of the five virulence genes were down-regulated 12 h after the 5% (w/v) Manuka honey treatment.

## 4. DISCUSSION

The results of this study show that Manuka honey significantly inhibited the growth of *P. brasiliense* at a sub-

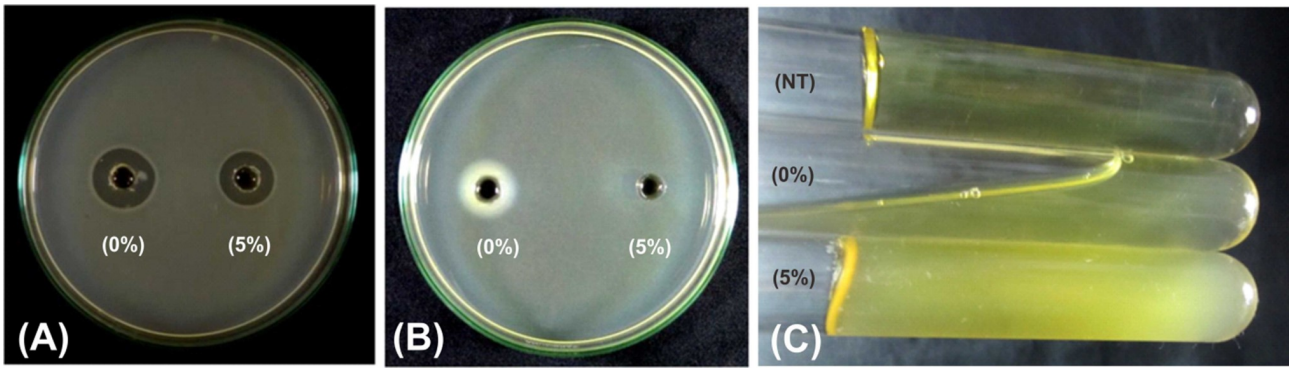


FIGURE 3. Extracellular enzyme production by *Pectobacterium brasiliense* in the presence of 5% or 0% (w/v) Manuka honey. (A) Pel; (B) Peh; (C) gelatin dissolution assay. Transparent zone and gelatin dissolution indicate enzyme production. NT, not treated gelatin without bacterial inoculation.

lethal concentration of 5% (w/v). Manuka honey decreased the number of bacterial cells, which resulted in lower growth curves compared with the untreated control. No bacterial growth was detected in cells treated with 10% (w/v) Manuka honey, suggesting that the compounds in Manuka honey hindered cell division and prevented bacterial growth. The first increase in the growth of the cells treated with 5% Manuka honey was observed at 10 h, whereas the growth of the untreated control (0% w/v) increased at 6 h. This result suggests that Manuka honey suppressed growth for 4 h. It has been confirmed that honey has a broad spectrum antibacterial properties, which may be attributed to the acidity (low pH), an osmotic effect, high sugar concentration, and the presence of bacteriostatic and bactericidal factors, such as hydrogen peroxide, MGO, flavonoids, peptides, and polyphenols (Israili 2014). Manuka honey is distinguished from other conventional honey by the unique Manuka factor (UMF) value which is indicated by the high concentration of MGO. The MGO concentration in Manuka honey is 38,761 mg/kg, which is 100-fold higher than that in conventional honey (Mavric et al. 2008). The decrease in bacterial growth was believed to occur due to the MGO content in Manuka honey, which adversely affects cell division. Cell copying and chromosome aggregation form proteinaceous rings (septum) that connect daughter cells. Peptidoglycan (murein) hydrolase degrades the cell walls between daughter cells, resulting in cell division (Priyadarshini et al. 2007). Inhibiting hydrolases hinders cell division. Manuka honey disrupts S. aureus cell division by inhibiting the activity but not the expression of murein hydrolase (Jenkins et al. 2011). Because of the high concentration of MGO in Manuka honey, MGO could be the main agent inhibiting the growth of *P. brasilience*. Several studies have reported that Manuka honey exhibits bactericidal activity against S. aureus (Almasaudi et al. 2017).

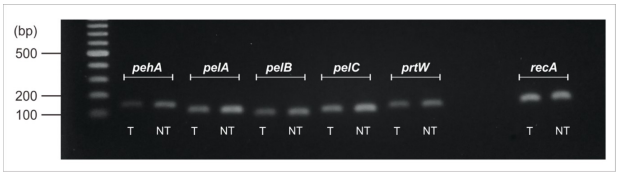


FIGURE 4. Expression of five virulence genes (*pehA, pelA, pelB, pelC,* and *prtW*) using two-step RT-PCR in *Pectobacterium brasiliense* with the recA gene as a positive control. The left side shows the 5% (w/v) treatment (T) with Manuka honey, whereas the right side is the non-treated (NT) 0% Manuka honey.

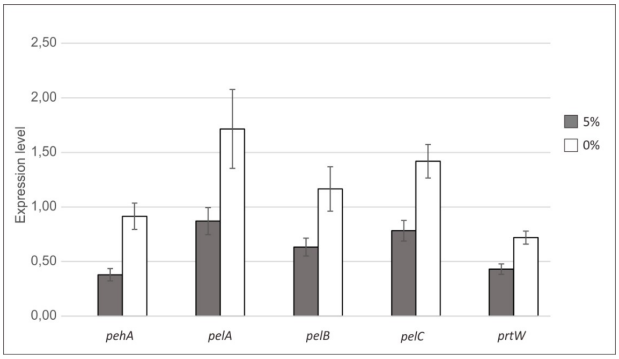


FIGURE 5. Semi-quantitative expression analysis of five virulence genes (*pehA, pelA, pelB, pelC,* and *prtW*) using two-step RT-PCR on *Pectobacterium brasiliense* with the *recA* gene as a positive control (bar heights and error bars represent the gene expression levels and standard errors, respectively).

Moreover, the Manuka honey treatment reduced the severity of soft rot on orchid leaves. This decrease in severity may be related to a decrease in bacterial virulence. This finding may be associated with decreases in Pel, Peh, and Prt production, which was confirmed by the plate assay, and the low gene expression of other genes that encode Pel (*pel*A, *pelB*, *pelC*), Peh (*peh*A), and Prt (*prt*W), the major virulence factors in *P. brasiliense*. Pel, which degrades plant cell walls, has profound effects on bacterial virulence. The combinations of pel gene clusters differ in various bacterial species; i.e., the pel gene cluster of *P. atrosepticum* consists of *pel*A, *pelB*, *and pelC*; that of *P. carotovorum* comprises *pel*A, *pelB*, *pelC*, and *pelD* (Zhang et al. 2017), whereas that of *Dickeya dadantii* consists of *pel*A, *pelB*, *pelC*, *pelD*, and *pelE* followed by four secondary genes, including *pelI*, *pelL*, *pelZ*, and *pelX* (Zhang et al. 2017). The Peh-encoding gene, *peh*A, affects bacterial virulence. The *P. carotovorum* pehA-mutant exhibits lower virulence when the pehA gene is deleted (Saarilahti et al. 1992). The *prt*W gene encodes metalloprotease (Mattinen et al. 2007), which also affects bacterial virulence.

The MGO content of Manuka honey inhibits enzyme production by affecting the expression of genes encoding the enzymes. The inhibitory effects of Manuka honey have been reported to reduce virulence, motility, and biofilm formation (Roberts et al. 2015). Extracellular enzyme production requires the plant signal N-(3-oxohexanoyl)-L-homoserine lactone, the QS signal, and several activating genes (Liu et al. 2008). Manuka honey inhibits jack bean urease activity through its MGO and dihydroxyacetone contents (Rückriemen et al. 2017). Jenkins et al. (2014) reported that 5% Manuka honey (two-fold the minimum inhibitory concentration) decreased the transcription of three genes on the *S. aureus agr* operon, which regulate

global virulence of the accessory gene regulator and the staphylococcal accessory regulator. The agr locus is a cluster of five QS genes (*agrA*, *agrB*, *agrC*, *agrD*, and *hla*). These genes produce and detect auto-inducing peptides which regulate the gene expression of virulence factors, such as extracellular proteases, hemolysin, surface-binding proteins, and biofilm formation.

## 5. CONCLUSIONS

The soft rot-causing bacterium *P. brasiliense* treated with 5% (w/v) Manuka honey exhibited lower virulence than the untreated control, as demonstrated by the decreased disease symptoms on orchids and the bacterial maceration ability. The Manuka honey treatment also reduced the production of extracellular enzymes (*Pel*, *Peh*, and *Prt*) and gene expression (*pel*A, *pelB*, *pelC*, *peh*A, and *prtW*), as detected by semi-quantitative PCR.

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## AUTHORS' CONTRIBUTIONS

SA, SS, MSR, NO, and TJ designed the study. SA conducted the experiments and analyzed the data under the supervision of TJ and SS. All authors have read and approved the final version of the manuscript.

## COMPETING INTERESTS

The authors declares no competing interests.

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