Jurnal Teknologi

Full Paper

RECOMBINANT PROTEIN APPLICATION INDUCED DEFENSE MECHANISM IN HOST PLANT AGAINST PAPAYA DIEBACK DISEASE

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Article history
Received
25 March 2023
Received in revised form
22 May 2023
Accepted
11 June 2023
Published Online
20 October 2023

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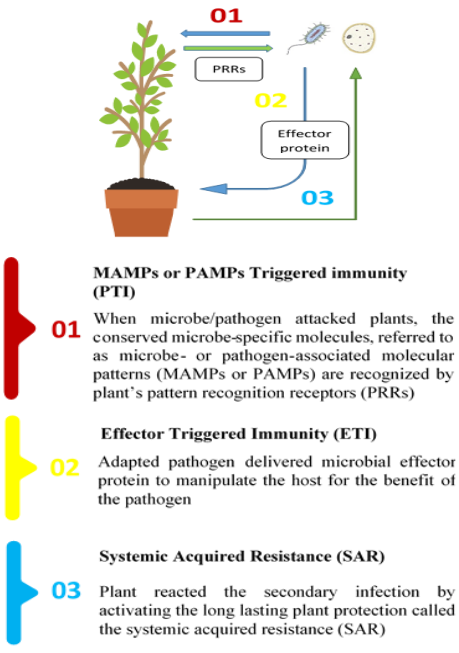
Graphical abstract



Three-layer of plant defence mechanism

Abstract

Erwinia mallotivora is a Gram-negative bacterium that causes the papaya dieback disease in Malaysia. Currently, no effective disease control method is documented. In this regard, the adoption of harpin proteins in promoting plant defense mechanisms has been reported to be a promising control method for this disease. This study used, two recombinant harpin proteins, Hrp I and Hrp II, to control the disease in the glasshouse and field conditions. The results of foliar application in the glasshouse showed protective index of 70.8% and 35.7% for Hrp I and Hrp II, respectively. Meanwhile, the field trial for Hrp I showed a promising protection index of 72.3%. Moreover, the plant growth-promoting attributes were also significantly improved in the case of the Hrp I -treated plants. The effectiveness of Hrp I and II as inducers of systemic acquired resistance (SAR) was further validated by profiling defense-related genes using RT-qPCR analysis. The results showed that the treated papaya plants with Hrp I possess the highest expression of defense genes such as peroxidase, osmotin, and PRID in the glasshouse and field samples. In conclusion, the application of Hrp I is a promising disease control approach in managing papaya dieback disease in Malaysia.

Keywords: *Erwinia mallotivora*, harpin protein, papaya dieback disease, systemic acquired resistance (SAR)

Abstrak

Erwinia mallotivora adalah bakteria Gram-negatif yang menyebabkan penyakit mati rosot betik di Malaysia. Buat masa ini, tiada kaedah kawalan berkesan telah dicatatkan. Oleh yang demikian, penggunaan protein *harpin* bagi menggalakkan mekanisme pertahanan tanaman dengan mendorong tindak balas tertentu telah dilaporkan sebagai kaedah kawalan yang berpotensi untuk penyakit tersebut. Dalam kajian ini, dua protein rekombinan *harpin*, Hrp I dan Hrp II, telah dipilih sebagai kaedah kawalan penyakit dalam ujian di rumah kaca dan lapangan. Keputusan

aplikasi foliar di rumah kaca menunjukkan indeks perlindungan sebanyak 70.8% dan 35.7% bagi protein Hrp I dan II. Keputusan ujian lapangan menunjukkan indeks perlindungan sekitar 72.3%. Tambahan pula, ciri-ciri penggalak pertumbuhan tanaman menunjukkan peningkatan ketara pada tumbuhan yang dirawat. Keberkesanan Hrp I and II sebagai pencetus ketahanan perolehan sistemik (SAR) telah divalidasi melalui pemprofilan gen berkaitan pertahanan melalui analisis RT-qPCR. Keputusan menunjukkan bahawa pokok betik yang dirawat dengan protein Hrp I memberi pengekspresan tertinggi gen-gen pertahanan seperti peroxidase, osmotin dan PRID untuk sampel rumah kaca dan lapangan. Sebagai kesimpulan, penggunaan protein Hrp I adalah pendekatan kawalan penyakit yang berpotensi untuk menguruskan penyakit mati rosot betik di Malaysia.

Kata kunci: *Erwinia mallotivora*, protein *harpin*, penyakit mati rosot betik, ketahanan perolehan sistemik (SAR)

1.0 INTRODUCTION

Crop production has significant hurdles due to pests and diseases. One of the most widely grown commercial fruit crops in tropical and subtropical climates is papaya [1]. However, the dieback disease caused by *Erwinia mallotivora* is the main issue restricting papaya production in Malaysia. Dieback disease causes wilting, discoloration of leaves, loss of plant turgidity, spots on foliage and petioles, water-soaked patches, premature fruit drop, and plant death [2]. Therefore, revitalizing the papaya industries in Malaysia requires the development of novel and effective control strategies for the disease.

The overuse of chemical pesticides is detrimental to the environment, ecology, and consumers [3]. Plants are known to possess an inherent self-defense mechanism. Systemic acquired resistance (SAR) is one of the most prevalent self-defense strategies used by plants. As an example of a natural plant defense mechanism, SAR protects plants from a several diseases [4]. It is a defense reaction that may be triggered in many plants, including papaya [5]. Salicylic acid (SA) is a naturally-occurring signaling molecule in plants that plays a significant role in plant defense against infection by different pathogens. It is essential for crop protection to produce recombinant protein-coded genes that can generate resistance to diseases. Recently, advances in biotechnology have allowed for the development of transgenic plants that express components of well-studied defense signaling pathways using SA analogs for SAR responses and the incorporation of other cutting-edge techniques, such as recombinant proteins and genes, with minimal to zero negative impact on the surrounding ecosystem. The application of recombinant harpin N (HrpN) protein isolated from *E. mallotivora* to papaya plants has been studied for its potential to induce a defense mechanism against dieback disease [6].

Disease-causing bacteria have developed effector molecules that could be translocated into their hosts and used to thwart the process of inducing plant resistance [7,8,9]. The interaction of these effector molecules with the host network and environment promotes the host defense mechanism. Different genera's pathogenicity relies on their secretion systems, which are complicated and fundamentally vital. This bacterium often infects and colonizes the apoplast or the gap between plant cells. The Hrp gene encodes a component of the plant pathogenicity machinery known as the type III secretion system (TTSS). A failure to produce Hrp in plants is caused by defects in the TTSS [10]. According to Kamoun [11], effectors are proteins and tiny compounds that produced by pathogens cause changes in the structure and function of the host cells which could aid infection or prompt a protective reaction. Most virulence and avirulence factors employ both methods. A major step forward in contemporary molecular plant bacteriology was the identification of HR and P genes shared by Gram-negative bacterial pathogens [12].

In 2017, a group of MARDI researchers including the authors have agrressively investigating this potential by adopting omics technology on *E. mallotivora.* The fundamental omics data had been revealed a long lists of effector proteins expressed from the bioinformatics study. The results obtained from pilot study found that, the tested two harpin protein which are recombinant protein Hrp I and Hrp II has developed the condition where can be considered as high potency in controlling dieback disease. Consequently, the selected effector protein of *E. mallotivora* was classified as a type III effector protein and utilised as recombinant protein under the harpin protein groups that reported to be useful and undergo a various plant defence related activity in papaya plant, this protein was chosen to be furthered study on their potential in creating biochemical events related to SAR mechanisms. Both

Hrp I and Hrp II are homologous and consist of similar active domain.

The present study aims to assess novel control strategies for managing papaya dieback disease by applying recombinant proteins that encode the Hrp I and Hrp II derived from *E. mallotivora* to activate the plant's defense mechanism. The outputs of this study will explore the potential use of the recombinant protein with encoded Hrp genes, as an inducer to plant defense mechanism that ultimately helps protect papaya from dieback disease infection.

2.0 METHODOLOGY

2.1 Preparation of Pathogens Suspension and Recombinant Protein Formulation

The pure culture of *E. mallotivora* strain BT-MARDI was provided by the Molecular Biology III Laboratory, MARDI, Serdang, Selangor. The bacteria (seven days old) were grown on LB plates and stored at 4 °C until use. A single colony of *E. mallotivora* was cultured in Luria Bertani (LB) broth and incubated at 28 °C in a shaking incubator for 6 hr, maintained at 200 rpm until the OD reached 1.0 at 600 nm. Cells were then harvested by centrifugation at 8000 rpm for 15 min and the bacterial pellet was washed twice with sterile distilled water (SDW). The harvested biomass was resuspended to obtain ~ 10^8 cfu/ mL bacteria.

The large-scale expression of Hrp I and Hrp II recombinant protein was conducted according to the pre-optimized protocol by Abu Bakar *et al.*, [6]. Briefly, the recombinant *E. coli* (BL21 DE3) harboring HrpI/pET-20b and HrpII/pET-32b plasmids were cultured in 1 L flasks containing 400 mL of LB supplemented with 400 µL ampicillin and agitated at 37° C for 1 hr until it reached mid-log phase at OD_{600}. Protein expression was induced by 0.05 M IPTG and incubated for 6 hr. The cells were harvested by centrifugation, and the pellet was resuspended in 10 mL inclusion bodies buffer with 0.5 % Triton and rotated at 4 °C for 15 min. The samples were then centrifuged for 30 min at 13,000 xg at 4 °C and the supernatant was removed. This step was repeated twice. The pellet was resuspended in 10 mL buffer and one protease inhibitor pill. The solution was circulated at 4 °C overnight and centrifuged 4 °C, 3,500 xg for 15 min. The supernatant was collected and purified using a Ni-NTA column via Acta Prime Chromatography System. The purified and concentrated proteins were diluted with SDW to the final desired concentration for the plant treatment.

2.2 Glasshouse Trial

A total of 120 of *Carica papaya* (Eksotika I) seedlings were grown in a glasshouse setting and watered twice daily. The trials were conducted as described by Abu Bakar *et al.*, [13], with slight modifications. Briefly, the two-month old papaya seedlings were treated with 10 mL of recombinant Hrp I and Hrp II proteins formulation at the concentration of 0.18 ng/L by foliar spray approach, while control seedlings were sprayed with SDW. The treatments were applied once a week for three weeks (days 1, 8, and 15).

The plants were inoculated with *E. mallotivora* suspension and SDW (negative control treatment) after a week from after the final treatment (day 22). Five mL of the bacterial suspension and SDW were injected once into the plant stem using a syringe with a fine needle[14]. Four combinations of treatments were set in this study (Table 1). Wilting symptoms and any changes in seedling leaf color were recorded in the one-month observation.

Table 1 Summary of plant treatments

Plant groups	Protein treatment (Day 1, 8 and 15)	Bacterial inoculation (Day 22)
1 (Negative control)	– (SDW)	– (SDW)
2 (Positive control)	– (SDW)	E. *mallotivora*
3	Hrp I	E. *mallotivora*
4	Hrp II	E. *mallotivora*

– (SDW): Protein treatment and E. *mallotivora* inoculation were replaced with sterile distilled water

2.3 Field Trial

A field trial was conducted to reaffirm the potential of recombinant protein in the open environment. This experiment was performed in two different open field areas in MARDI: i) papaya dieback disease hotspot area and ii) non-hotspot area. The hotspot area is where the elevated disease and higher probabilities of disease occurred [15]. The experiment was repeated twice at the same location, from January to September, for two consecutive years. The standard agronomic practices of soil management (good drainage system and fertilization program), besides pest and disease management recommended by MARDI, were adopted. Two treatments were applied: control plants without treatment and plants treated with recombinant Hrp I, applied via foliar spray on the whole plant leaf surfaces. The frequency, volume, and dose of recombinant protein Hrp I sprayed during treatment are shown in Table 2. The application was done at monthly intervals for up to nine months. Similar treatments were applied in the non-hotspot area. The symptoms of papaya dieback disease were recorded for nine consecutive months.

Table 2 Volume and dosage of recombinant protein Hrp I application based on month application

Months after treatment	Volume (mL) / plant	Concentration (µg/mL) / plant
1	10	0.4
2	10	0.4
3	20	0.6
4	20	0.6
5	30	0.8

Months after treatment	Volume (mL) / plant	Concentration (µg/mL) / plant
6	40	1.0
7	40	1.0
8	40	1.2
9	40	1.2

2.4 Disease and Plant Growth Assessments

The disease assessment data was recorded for the glasshouse and field trial plants. The disease incidence (DI) was determined as the number or proportion of diseased plant units relative to the total number of units tested [16]. The disease severity index (DSI) was generated according to papaya dieback disease scales created by Abu Bakar *et al*., [6] ; scale 0 = healthy/no symptom, 1 = leaf vein blackening, 2 = leaf vein blackening and slightly wilting, 3 = leaf stalk wilting, 4 = stem blackening, and 5 = plant death.

The area under disease progress curve (AUDPC) and protection index (PI) for disease incidence (DI) were determined using a formula provided by Groth *et al*., [17] and Campbell and Madden [16].

The growth evaluation in field trial plants included three main parameters: plant height, stem diameter, and the number of leaves. Data were collected once a month for nine-consecutive months for each experiment. The plant height was measured from the base of the plant at the ground surface to the top of the youngest fully expanded leaf using a measuring tape. The number of leaves per plant primarily attached to the main stem or petiole was counted manually. Vernier caliper (0.02 mm) was used to measure the diameter of the main stem 15 cm from the ground [18]. The number of fruits was counted per plant, and the fruits were harvested for fruit weight. Data of fruit numbers of each plant in the field were scored and recorded for nine months after planting. The fruit count included the young fruits formed just after anthesis. Ten mature fruits at the maturity index 2 (green skin with a light yellow stripe) from each plant were harvested, and the mean weight of fresh fruit was recorded. Meanwhile, the yield was calculated from the product of the mean fruit number and fruit weight.

2.5 Experimental Design and Data Analysis

Randomized complete block design (RCBD) was applied for the glasshouse and field trial experiments, with n = 20 and n = 60 replicates for each treatment. Means comparison of the treatments for glasshouse and field data were separated using one-way ANOVA (Tukey's Honest Significant Difference) at $P \leq 0.05$ using SAS software.

2.7 Profiling the Expression of Plant Defense Genes

The leaves of papaya plants were collected from the glasshouse and field plots (hotspot area). The plant samples were labeled based on plant disease assessment as follows; C= Control (Untreated healthy) plants, T1 = Treated healthy plants, T2 = Untreated infected plants, and T3 = Treated infected plants. Each sample has three biological replicates to show biological variation. The samples were stored in liquid nitrogen, ground into fine powder and kept in the -80 °C freezer for further usage.

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Germany), according to the manufacturer's protocol. The integrity of the extracted total RNA was determined using 1% agarose gel electrophoresis, and the quality was assessed using Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, USA). The synthesis of cDNA from total RNA was conducted using Reverse Transcription System Kit (BioRad, USA), according to the manufacturer's protocol.

The RT-qPCR was performed in the Bio-RAD CFX96 Real-Time PCR System Thermocycler using the SensiFAST SYBR Hi-ROX kit from Bioline, USA (Bio-Rad, USA. The genes of interest were peroxidase, osmotin, and PRID [19], with actin and 40sRP [20] as the reference genes. The relative expression ratios of the genes of interest in healthy treated (T1), infected (T2 and T3), and healthy control samples (C) were estimated from the acquired Cq values using the 2-CT technique [21].

3.0 RESULTS AND DISCUSSION

3.1 Glasshouse Trial on the Effectiveness of Harpin Protein Treatment

Disease Symptomatology and Incidence

Figure 1 depicts the symptomatology of infected papaya seedlings inoculated with *E. mallotivora*. Changes are observed in the plant phenotype, which can be described in five stages of disease development, as reported by Abu Bakar *et al.,* [6].

Figure 1 Development of dieback disease on infected papaya seedlings after inoculated with EM: (A) healthy leaves (B) first stage (C) second stage (D) third stage, (E) fourth stage, (F) fifth stage of papaya dieback disease

Figure 2 depicts the results of disease incidence. Since this study was conducted under the controlled

environment of a glasshouse, the results of control untreated papaya seedlings inoculated with *E. mallotivora* generated nearly 100% disease incidence. This incidence is true for Eksotika as this papaya variety is highly susceptible to papaya dieback disease [24, 25]. The disease incidence decreased tremendously in papaya treated with recombinant Hrp I and Hrp II. Figure 2 shows that 50% of the disease occurred in papaya seedlings treated with Hrp I, while 60% was detected in papaya seedlings treated with recombinant protein Hrp II. The outcome is affected by the treatment used. Tukeys HSD analysis of the data reveals a significant difference between the treated and untreated *E. mallotivora* inoculated plants (Figure 2).

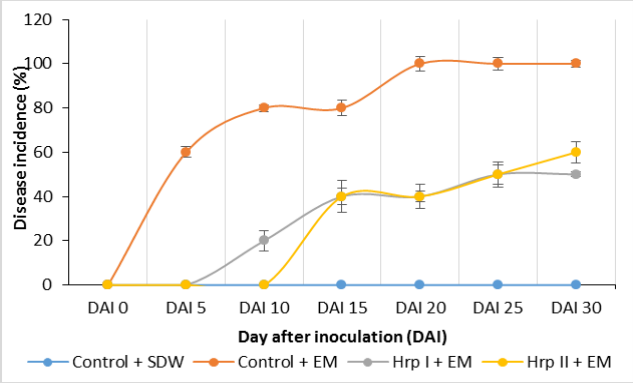


Figure 2 Disease incidence of papaya plants in glasshouse trial

Disease Severity

The disease assessment performed in this experiment and the outcomes of disease severity are illustrated in Figure 3. Statistical study reveals a substantial difference between untreated and treated papaya seedlings with recombinant protein Hrp I and Hrp II.

The highest disease severity of plant inoculated with *E. mallotivora* at 30 days after inoculation (DAI) was recorded in the control untreated plant (99.8%), followed by plants treated with recombinant protein Hrp II (68.9%) and Hrp I (35.4%).

The earliest symptom was observed at 5 DAI in the untreated control plant inoculated with E. *mallotivora*, while the plant treated with recombinant protein Hrp II (15 DAI) and Hrp I (10 DAI) inoculated with E. *mallotivora* showed delayed symptoms development.

On 25 DAI, the leaves of the Hrp I-treated plant inoculated with E. *mallotivora* began to yellow and withered, followed by the fall of infected organs, finally leading to the plant's survival. These data suggest that recombinant Hrp I treatment induced the defense mechanism in infected papaya seedlings and elicited the HR response to localize the infection and increase disease resistance toward *E. mallotivora.* This is aligned with Bocscanzy *et al*. [22], who revealed that harpin protein would enhance the plant's defense mechanism through SAR activation, thereby permitting the plant to engage in HR, which aids in the localization and removal of infected tissues.

The disease severity in Hrp II-treated plants inoculated with *E. mallotivora* is decreased by 4.6% on 25 DAI, and the plants start to become 50% more vigorous. The infected parts started to fall, and the plants demonstrated a favorable disease reduction and were suspected to have increased their immunity. It is similar to Amil-Ruiz *et al*. [23], who reported that recombinant Hrp II delayed the infection and increased the defense system. Unexpectedly, after 30 DAI, the plants' withering intensified, with the leaves turning yellow, the veins darkened, and the petiole bent downward, ending in plant death.

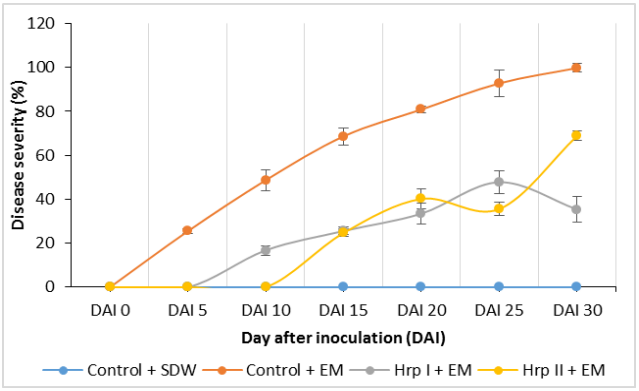


Figure 3 Disease severity of papaya plants in glasshouse trial

Area Under Disease Progress Curve (AUDPC) and Protection Index (%)

The papaya seedlings treated with recombinant proteins Hrp II and I and inoculated with *E. mallotivora* showed the highest AUDPC, with values of 150 units^2 and 110 units^2. The control papaya seedlings inoculated with *E. mallotivora* showed the highest AUDPC, with a value of 180 units^2. The papaya seedlings treated with recombinant Hrp I had the lowest AUDPC value. In this study, the AUDPC value was assessed to identify pathogen intensity in papaya seedlings over time after exposure to different treatments. The untreated papaya seedlings (control) showed the highest AUDPC value after inoculation with *E. mallotivora*, indicating that the pathogen proliferated rapidly and resulted in the complete death of the papaya plants.

The PI value of recombinant Hrp I was the greatest at 70.8%, while the value of recombinant Hrp II was 35.7%, and no PI value was recorded for the control and untreated papaya seedlings. All tabular information can be found in Table 3. The PI was employed to quantify the efficacy of the treatments relative to the control. The highest PI by recombinant Hrp I is regarded as the most effective

treatment for controlling infected papaya plants compared to recombinant Hrp II.

Table 3 Effects of foliar application using recombinant Hrp I and Hrp II

Treatment	AUDPC (Unit²)	Protection Index (%)
Control + SDW	0	0
Control + EM	180	0
Hrp I + EM	110	70.8
Hrp II + EM	150	35.7

The effectiveness of harpin protein in decreasing the severity of the disease, producing high PI, and reducing AUDPC is well documented in many studies. Different harpin proteins had varying degrees of potency in reducing the severity of the disease on plants [26]. For instance, it has been reported that the protein fragment encoding genes that induce defense mechanism, such as HpaG10-42, comprising 10–42 amino acids, can induce disease resistance in rice by reducing the severity of the disease 1.5 and 7.5 times more effectively than HpaGXooc [27]. It is congruent with this study, whereby the Hrp I protein encoding the hrp genes that induce SAR by reducing disease severity and incidence will lead to disease resistance in papaya seedlings toward *E. mallotivora* infection.

3.2 Field Trial Efficacy of Hrp I Recombinant Protein

The symptomatology of the non-hotspot area for treated and untreated papaya trees (control) demonstrated that the papaya trees were free from dieback disease and showed no disease symptoms. The papaya trees were planted in a non-hotspot area with no *E. mallotivora* inoculum.

Meanwhile, the plants with the control treatment in the hotspots area were severely infected with papaya dieback disease. Typical disease symptoms on the infected plants were recorded on the stem, leaves, petioles, and fruits. The infection was first observed three months after treatment. The recorded field disease symptoms were similar to those reported by Mohd Azhar *et al*. [25]. This study also revealed that the dieback disease infection and severity are more predominant and rapid once the plant reaches the flowering and fruiting stages. According to Maktar *et al*. [28], insects and birds are dissemination agents or vectors that spread the pathogen from one plant to another. Hence, these vectors actively spread the disease during the flowering and fruiting stages, indirectly causing rapid dieback outbreaks in the hotspot areas.

The symptomatology of the plants treated with Hrp I is presented in Figure 4. The first disease symptom was observed five months after treatment, i.e., minor symptoms with discoloration and patches of necrosis on the infected leaves (Figure 4B and C), while other organs remained symptom-free. Figures 4D, E, and F illustrate the progression of leaf yellowing and vein darkening symptoms. Consequently, the infected leaves became blackened, and variegated patches appeared. This is consistent with Mat Amin *et al*. [29], who reported blackened leaves and stems and greasy spots observed at the infection sites.

During the observation, disease infection was recorded only at the first and second stage infection, where no further disease progression was recorded. This finding suggests the role of Hrp I in activating the defense mechanism to limit disease development or further invasion and colonization of pathogens, thereby reducing disease intensity and spread [30].

This agrees with the result of this study, which showed that the infected papaya leaves turned yellow, showed necrotic lesions, and eventually fell off from the papaya plants, and the dieback disease did not progress to the other parts of the plant. It is also thought to be the quarantine zone for the biotrophic pathogens that hamper further pathogen spreading towards other healthy tissue cells [31].

The formation of necrotic lesions is seen as the manifestation of hypersensitive response (HR) that contains rapid collapse of tissues. It has been reported that SAR can be induced by various factors, including exogenous effectors [6], where the Hrp I protein is one of the effector proteins obtained from the omics and bioinformatics study (unpublished data).

The application of effector protein noticeably blocked the advance of the pathogen in papaya plants. It signifies that recombinant protein Hrp I can aid the defense mechanism by eliciting the HR, which induces SAR in treated papaya plants.

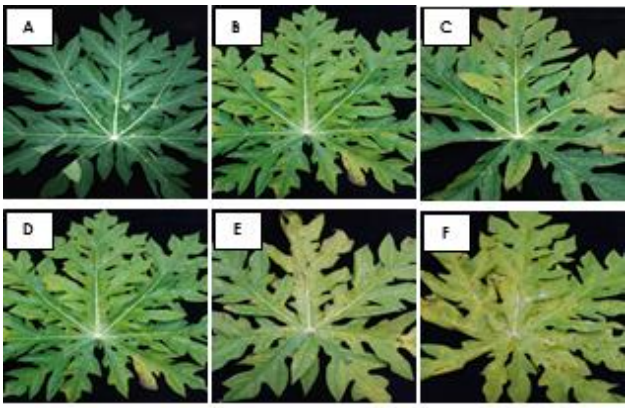


Figure 4 Disease progression in the Hrp I treated papaya leaves. A) No symptom; B to F) Mild symptoms of PDD. There are no symptoms reported at other parts of plants

Disease Incidence

The results of disease incidence are presented in Figure 5. About 100% of disease incidence is recorded in control untreated plants at the hotspot area. In contrast, both the untreated and treated with recombinant Hrp I planted control plants in the non-hotspot area were free from disease, as

expected (no disease incidence was recorded). In summary, only 20% of disease incidence is detected in papaya plants treated with recombinant protein Hrp I compared to 100% in the control plants. The application of recombinant Hrp I protein to papaya plants managed to decrease 80% of disease incidence compared to control papaya plants. Figure 5 shows a significant difference between the treated and untreated plants.

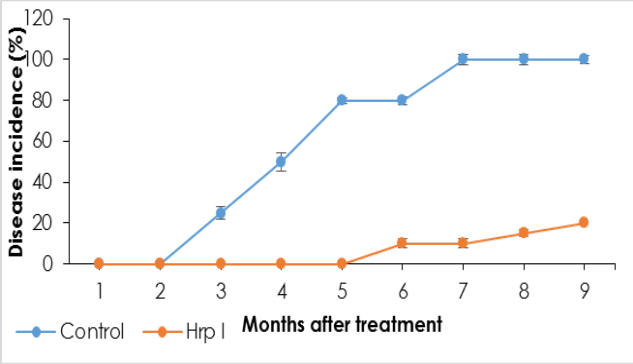


Figure 5 Disease incidence of papaya plants in field trial

Disease Severity

Findings of the present study revealed that disease severity of the control untreated plants are 0%, 27.8%, 49.7%, 69.7%, 78.9%, 89.8%, 92.7%, and 100% at 0–2, 3, 4, 5, 6, 7, 8, and 9 months after treatment. Meanwhile, the treated plants demonstrated the lowest disease severity of 0%, 18.3%, 27.3%, 29.8%, and 33.6% at 0–5, 6, 7, 8, and 9 months after treatment. Details of the results are presented in Figure 6.

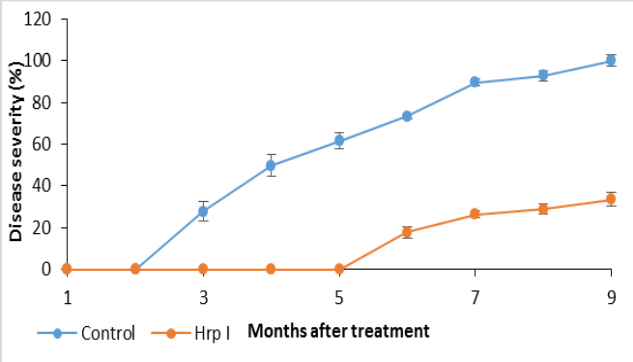


Figure 6 Disease severity of papaya plants in field trial

Papaya plants treated with recombinant protein Hrp I displayed reduced disease severity by 66.4% compared to the control plants. This finding demonstrates that foliar application of recombinant protein Hrp I could reduce disease severity. Moreover, the recombinant protein Hrp I functions well in mimicking the pathogen infection situation and activates the SAR mechanism as plant defense in treated papaya plants. The expression of plant defense genes enables the papaya plant to combat infection, leading to disease resistance and reducing the severity of infection [32].

Area Under Disease Progress Curve (AUDPC) and Protection Index (%)

Untreated papaya plants in the hotspot area demonstrated the highest AUDPC, with a value of 308.9 unit^2, followed by plants treated with recombinant protein Hrp I at 103.7 unit^2. The untreated papaya plants demonstrated the highest AUDPC value, indicating that the pathogen/disease has been multiplying aggressively and causing the total loss of papaya plants and yield.

The PI of the treated plant with recombinant Hrp I recorded the highest PI value of 72.3%. The tabulated data can be referred to in Table 4. The PI was used as a measurement of the effectiveness of the treatments compared to the control. In this study, the highest PI by recombinant Hrp I is considered the most effective treatment to control papaya dieback.

Table 4 Effects of foliar application using recombinant Hrp I on *E. mallotivora* infected papaya plantlets

Plot	Treatment	AUDPC (Unit^2)	Protection Index (%)
Non-hotspot area	Control	0	0
	Hrp I	0	0
Hotspot area	Control	308.9	0
	Hrp I	103.7	72.3

Plant Growth Assessment

The changes in the height of papaya plants were observed every four weeks. The height of the plant was recorded for seven consecutive months (3 to 9 months). The development of plant height is presented in Figure 7. The plant height significantly increases from week four to the final stage of plant growth (fruiting stage). Significant differences are recorded in the hotspot area at 7- and 9-month assessment periods.

The height of untreated control and treated with recombinant Hrp I papaya plants in the non-hotspot area reached 178.5 cm at 9 months. Meanwhile, the height of the untreated control papaya plant in the hotspot area reaches 142.8 cm. The papaya plant treated with recombinant Hrp I protein in the hotspot area reached 179.5 cm in the same assessment period.

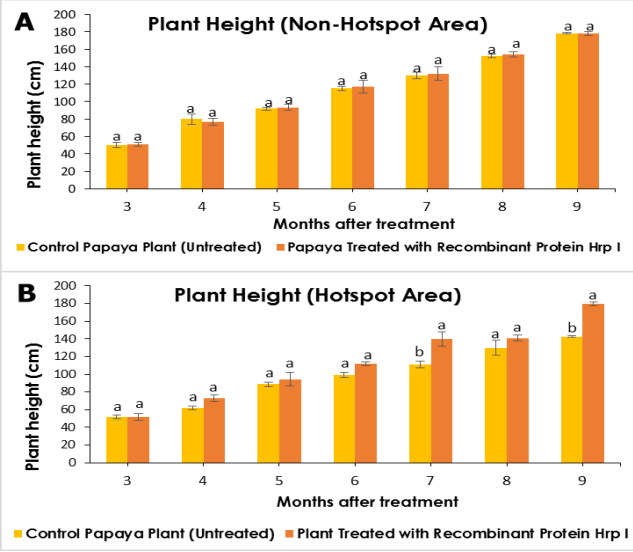


Figure 7 Plant height of papaya plants where, (A) non-hotspot and (B) hotspot area. Data are means of triplicate measurements with standard error bars. Means with different letters between treatments within the same months indicate significant differences at p≤0.05

The stem diameter of papaya plants was measured once a month, and the results are recorded in Figure 8. The plant height was also recorded in this study. The stem diameter of the untreated control and treated with recombinant Hrp I papaya plants in the non-hotspot area reached 25.6 cm at 9 months. The untreated control and treated with recombinant Hrp I protein papaya plant in the hotspot area reached 20.3 and 24.8 cm at the assessment time. Statistically, no significant difference in stem diameter was recorded between treatments up to five months. Whereas significant differences recorded from 6-month assessment periods.

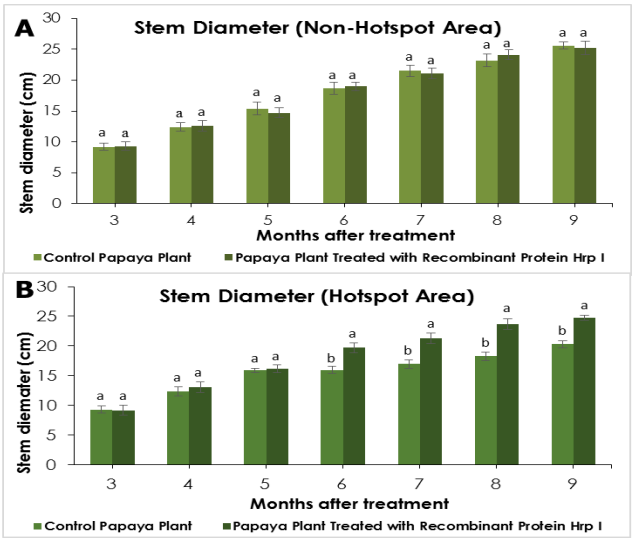


Figure 8 Stem diameter of papaya plants where, (A) non-hotspot and (B) hotspot area. Data are means of triplicate measurements with standard error bars. Means with different letters between treatments within the same months indicate significant differences at p≤0.05

One of the fundamental measurements for plant development is the number of leaves. The number of leaves indicates the growth rate condition of the plants. The control papaya plants in the hotspot area reduced their leaf production once infected by the pathogen. The data are illustrated in Figure 9.

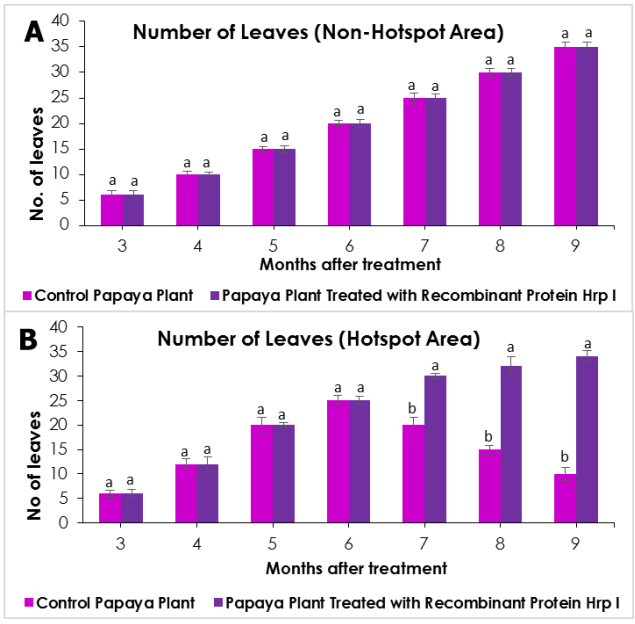


Figure 9 Number of leaves of papaya plants where, (A) non-hotspot and (B) hotspot area. Data are means of triplicate measurements with standard error bars. Means with different letters between treatments within the same months indicate significant differences at p≤0.05

Figure 9 shows that the number of leaves for untreated control and treated papaya plants in the non-hotspot area is similar. However, the hotspot area depicts a different trend. Infected papaya plants stopped producing new leaves and shoot due to bacterial colonization in the xylem and phloem, which disturbs photosynthesis [33]. The number of leaves for untreated control plants decreased at the age of 7 months from 25 to 20 leaves and further declined to 10 leaves at 9 months (Figure 9). This is aligned with Mat Amin *et al.* [29] on the progress of papaya disease, which causes the leaves to discolor, blacken, and fall. The statistical analysis indicates a significant difference in untreated control and treated papaya plants at 7 to 9 months assessment periods.

The ability of the treatment to induce disease resistance in host crops would increase production yield and crop quality. Table 5 depicts the yield and its components (fruit number and fruit weight), which are often correlated with plant health. The results indicate a significant difference between untreated control and treated papaya plants in terms of the number of fruits, fruit weight, and papaya output per plant in the hotspot area.

Table 5 Number of fruits counted, fruit weight and yield of papaya plants

Plot	Treatment	Fruits/ plant	Weight (g)/ fruit	Yield (kg)/ plant
Non-Hotspot area	Control	24[a]	510.8[a]	12.4[a]
	Hrp I	23[a]	508.9[a]	13.1[a]
Hotspot area	Control	16[b]	430.4[b]	7.9[b]
	Hrp I	24[a]	512.7[a]	13.8[a]

Note: All data are analysed using Tukey's Honest Significant Difference (HSD). The, means within the column with the same letters indicated no significance difference (P>0.05)

The number of fruits for untreated control papaya plants is lower than treated papaya plants, i.e., 16 and 24 fruits/plant. The results are comparable in terms of fruit weight; untreated control papaya plants produced lesser fruit weight (430.4 g), while treated papaya plants produced between 508.9 to 512.7 g. Moreover, the maximum output for this component is just 7.9 kg/plant for untreated control papaya plants compared to 13.8 kg/plant for treated papaya plants. This finding showed that recombinant Hrp I can promote productivity. Statistical analysis indicates that both treatments are grouped under different groups, which is significantly different between groups. Fallahi [34] also found that the apple fruit from trees that received harpin protein had significantly (about 23%) better color, earlier ethylene development, and higher respiration than those from untreated control trees. The application of Hrp I could generate substantially more papaya fruits than the control treatment. However, it is still unclear how recombinant protein affected the productivity of the plant. However, it is safe to speculate that the proteins may have favorably impacted plant chloroplasts [35]. It is hypothesized that the chloroplast would be the primary target of effector proteins since it is crucial for photosynthesis [36]. Photosynthesis uses energy from light to convert water and carbon dioxide molecules into glucose (sugar molecule) and oxygen, and when oxygen is released or "exhaled" from the leaves, the energy contained within the glucose molecules is used throughout the plant for growth, flower formation, and fruit development [37]. This mechanism cleared a fragment of how harpin affects photosynthesis and is related to fruit production.

3.4 Validation of Plant Defense Genes Expression via RT-qPCR

The symptomatological results for recombinant Hrp I and Hrp II treatments yielded positive results in the control of papaya dieback disease. This event was anticipated as SAR was induced and the plant's defenses were activated.

However, the symptomatological data obtained were insufficient to prove the induction of the SAR mechanism, and the role of recombinant Hrp proteins in papaya defense also remained unclear. Therefore, an expression profile of three selected defense-related genes (peroxidase, osmotin, and PRID) was performed, chosen for their roles in the plant defense system. In the analysis of the results, the expression ratio of ≥ 2-fold was defined as the cut-off point where an expression ratio higher than this was considered differentially expressed [38]. All gene expression folds described here were relative to their respective control sample (untreated-healthy) in each experimental series.

Peroxidase Gene

For the peroxidase gene (Figure 10a), no induction (~1-fold) was observed in Hrp I- and Hrp II-treated healthy plants (red bars) compared to the control (untreated healthy) in both trial locations (glasshouse and field).

A slight induction (>1.8-fold) of this gene is observed in untreated infected plants (green bar), with the highest induction (2.17-fold) occurring in the field sample. This finding indicates that the induction of plant defense genes is initiated when the specific receptors recognize the presence of *E. mallotivora* attacks. This result is consistent with the study by Diaz [39], which found that the plant defense system is only activated upon and during infection by pathogens, pests, or abiotic stress.

Significant upregulation of the peroxidase gene was observed in Hrp I-treated infected plants (purple bar), with an expression level of 2.6- and 3.6-fold for the glasshouse and field samples. Meanwhile, infected plants treated with Hrp II retained their expression (~2-fold) without recombinant protein treatment.

Osmotin

In addition to peroxidase, the osmotin gene is also involved in papaya's defense mechanism. Figure 10b depicts healthy plants (red bar) treated with Hrp I and II showing a slight insignificant increase (1.7- to 1.8-fold) in osmotin expression. Meanwhile, no induction of osmotin expression (~1-fold) is observed in healthy plants treated with Hrp II. This non-induction/insignificant upregulation of osmotin could be due to the pathway of the plant defense mechanism, which is only activated when a signal for pathogen attack is received.

A significant upregulation (2.5-fold) of osmotin is seen in the field sample of untreated infected samples (green bar), while no significant induction (1.5-fold) is observed in the glasshouse sample. The osmotin gene expression in infected field papayas is higher than in the greenhouse, possibly due to the uncontrolled environment, including abiotic stress factors like temperature and soil conditions. Overexpression of the osmotin gene due to salt stress was detected in transgenic strawberry plants (*Fragaria x ananassa*) [46], while transgenic tomato (*Solanum lycopersicum*) plants expressing tobacco

osmotin gene showed enhanced expression of various stress-responsive genes when exposed to cold stress [40].

Osmotin expression is significantly up-regulated in the Hrp I-treated infected plants (purple bars), with 2.3- and 3-fold expression in the glasshouse and field samples. In contrast, a slight increase (1.9-fold) of osmotin is detected in the infected Hrp II-treated sample.

Viktorova *et al*. [41] reported that osmotin is important in the plant immune system during stress. Likewise, Bashir *et al*. [42] stated that osmotin is a cationic protein that improves biotic and abiotic stress tolerance in plants. Furthermore, since this experiment was conducted in an uncontrollable environment for about nine months per experiment, it is logical that, in addition to *E. mallotivora* infection, abiotic factors such as temperature and weather influence the expression of the defense genes. According to Alhaithloul [43], the cellular protein osmotin is expressed in plants in response to heat or high-temperature stress. Apart from that, osmotin is also involved in the initiation of apoptosis and programmed cell death, and its overexpression causes the accumulation of proline in transgenic plants [44]. Likewise, the symptomatic results showed that papaya treated with recombinant protein Hrp I could survive by locating the site of infection and died without disturbing non-infected parts of the plants.

Pathogenesis-Related Genes (PR-ID)

Pathogenesis-related genes (PRID) are important genes that exist in the plant defense system. Profiling of PRID (Figure 10c) showed no significant induction of the gene in all treated healthy (red bar) and untreated infected (green bar) samples in all experimental series. However, PRID was significantly up-regulated in the Hrp I-treated infected plants, with a 2.3-fold expression in the greenhouse samples and 3.6-fold in the field samples. However, PRID expression remained at its basal level in infected plants treated with Hrp II.

PRID belongs to the group of PR proteins and reportedly plays multiple roles in adaptation to abiotic stress. Dana *et al*. [45] reported that transgenic tobacco overexpressing a PR gene that induced endochitinase improved tolerance to both biotic (*Pseudomonas syringae*) and abiotic (salt and heavy metals) stress. Another study also found that overexpression of PR-5 protein in rice increases tolerance to *Rhizoctonia solani* [46], while overexpression of capsicum PR-1 in tobacco increases host tolerance to *Phytophthora nicotianae*, *Ralstonia solanacearum*, and *P. syringae* [47].

PRID was also overexpressed when the infected papaya samples were treated with recombinant protein Hrp I. Similarly, Linthorst [48] and Cutt and Klessig [49] reported that when plants try to resist a pathogen attack, it also synthesizes several PR proteins. Hence, the overexpression of PR genes is required to uplift the level of defense response in papaya against *E. mallotivora*. Ali *et al*. [50] revealed that overexpression of PR genes individually or in combination has greatly increased the defense mechanism in plants against various pathogens. This is demonstrated in the overexpression of osmotin and PRID in infected papaya, which is a good combination in increasing the plant defense to resist dieback disease. PRID also helps inhibit *E. mallotivora* from aggressively infecting papaya. Similarly, Dzhavakhiya *et al*. [51] reported that the combination of two or more PR proteins may inhibit pathogen growth.

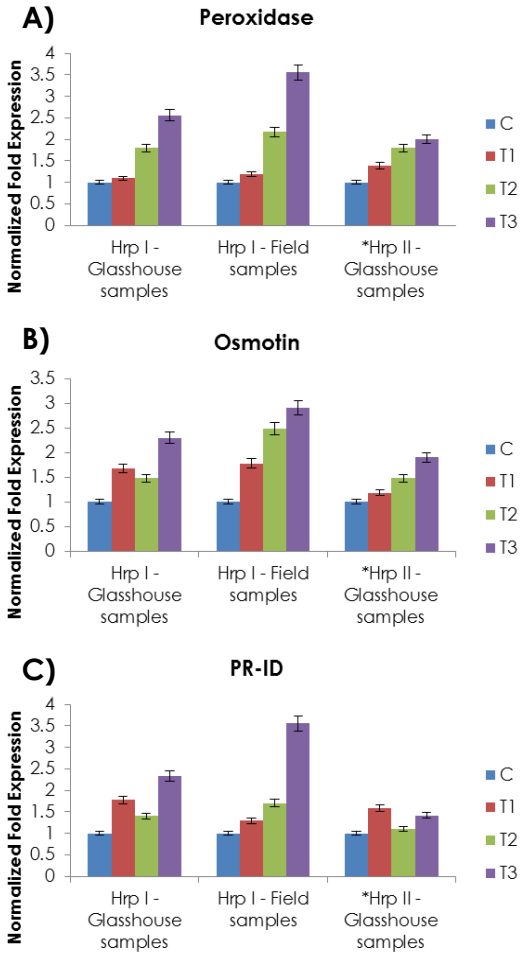


Figure 10 Normalized fold expression of A) peroxidase, B) osmotin and C) PR-ID genes from glasshouse and field samples. C: Healthy control samples, T1: Treated healthy samples, T2: Untreated infected samples, T3: Treated infected samples

This profiling study showed a significant upregulation of all candidate genes was consistently demonstrated in Hrp I-treated plants with *E. mallotivora* infection. The plant defense system induces SAR upon a pathogen attack. According to Durner *et al*. [52] and Hu *et al*. [53], plants often wait until pathogens are detected before producing defense-related proteins due to the high-energy and

nutrient requirements associated with protein production. Similarly, De León and Montesano [54] agree that signaling pathways are activated once the stress is sensed, leading to the induced expression of genes with different roles in defense. This explains why the treatment of harpin protein itself does not trigger the defense gene expression in healthy plants.

4.0 CONCLUSION

The recombinant Hrp I is more effective than the recombinant Hrp II in inducing SAR and displaying higher disease resistance. Furthermore, the recombinant protein treatment in the field study proved that the recombinant Hrp I can reduce the disease severity of infected plants without affecting their growth besides increasing fruit production. RT-qPCR confirmed that the recombinant proteins could induce SAR and develop a hypersensitive response which enables local and rapid cell death to confine and prevent the spread of the pathogen.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

Acknowledgement

This research is fully supported by the Malaysia Agriculture Research and Development Institute (MARDI) Development grant. The authors fully acknowledged MARDI and Universiti Putra Malaysia for the approved fund, which makes this important research viable and effective.

References

[1] Chávez-Pesqueira, M., and Núñez-Farfán, J. 2017. Domestication and Genetics of Papaya: A Review. *Front Ecology Evolution.* 5(155).
Doi: https://doi.org/10.3389/fevo.2017.00155.

[2] Sekeli, R., Nazaruddin, N., Tamizi, A., Amin, N., Wee, C.-Y., Sarip, J., and Zulkifli, Z. 2019. Enhancing Eksotika Papaya Resistance to Dieback Disease through Quorum Quenching. *Journal of Tropical Plant Physiology*. 11(1): 1-9.

[3] Sharma, A., Kumar, V., Shahzad, B., Tanveer, M., Sidhu, G., Handa, N., and Thukral, A. 2019. Worldwide Pesticide Usage and Its Impacts on Ecosystem. *SN Applied Sciences.* 1(1446).
Doi: https://doi.org/10.1007/s42452-019-1485-1.

[4] Reynolds, G., Gordon, T., and McRoberts, N. 2019. Using Game Theory to Understand Systemic Acquired Resistance as a Bet-Hedging Option for Increasing Fitness When Disease Is Uncertain. *Plants*. 8(7): 219.
Doi: https://doi.org/10.3390/plants8070219.

[5] Samsuddin, N., Abu Bakar, N., Shaharuddin, N., Azzeme, A., Laboh, R., Badrun, R., and Muhamad Nor, A. 2018. Establishment of Systemic Acquired Resistance (SAR) in Papaya by External Salicylic Acid Application as a Strategy to Control Dieback Disease. *International Journal of Current Research*. 10(10).
Doi: https://doi.org/10.24941/ijcr.32696.10.2018.

[6] Abu Bakar, N., Sohaime, M., Juri, N. M., Badrun, R., and Sarip, J. 2018. Induction of Systemic Acquired Resistance in Papaya by Foliar Application of HrpN Recombinant Protein for Increased Resistance against Papaya Dieback Pathogen. *Current Investigations in Agriculture and Current Research*. 2(3).
Doi: https://doi.org/10.32474/CIACR.2018.02.000136.

[7] Todd, J. N., Carreón-Anguiano, K. G., Islas-Flores, I., & Canto-Canché, B. 2022. Microbial Effectors: Key Determinants in Plant Health. *Microorganisms*. 10(10): 1980.
Doi: https://doi.org/10.3390/microorganisms10101980.

[8] Zhang, S., Li, C., Han, Z., and Chen, D. 2022. Action Mechanisms of Effectors in Plant-Pathogen Interaction. *International Journal of Molecular Sciences*. 23(12): 6758.
Doi: https://doi.org/10.3390/ijms23126758.

[9] Jiang, Y., Zhang, C.-X., Chen, R., and He, S. 2019. Challenging Battles of Plants with Phloem-feeding Insects and Prokaryotic Pathogens. *Proceedings of the National Academy of Sciences (PNAS)*. 116(47): 23390-23397.
Doi: https://doi.org/10.1073/pnas.1915396116.

[10] Vander Broek, C., and Stevens, J. 2017. Type III Secretion in the Melioidosis Pathogen *Burkholderia pseudomallei*. *Frontiers in Cellular and Infection Microbiology*. 7: 255.
Doi: https://doi.org/10.3389/fcimb.2017.00255.

[11] Kamoun, S. 2007. Groovy Times: Filamentous Pathogen Effectors Revealed. *Current Opinion in Plant Biology*. 10(4): 358-365.
Doi: https://doi.org/10.1016/j.pbi.2007.04.017.

[12] Pfeilmeier, S., Caly, D. L., and Malone, J. G. 2016. Bacterial Pathogenesis of Plants: Future Challenges from a Microbial Perspective: Challenges in Bacterial Molecular Plant Pathology. *Molecular Plant Pathology*. 17(8): 1298-1313.
Doi: https://doi.org/10.1111/mpp.12427.

[13] Abu-Bakar, N., Juri, N. M., Abu-Bakar, R. A. H., Sohaime, M. Z., Badrun, R., Sarip, J., Hassan, M. A., and Ahmad, K. 2021. Recombinant Protein Foliar Application Activates Systemic Acquired Resistance and Increases Tolerance against Papaya Dieback Disease. *Asian Journal of Agriculture and Rural Development*. 11(1): 1-9.
Doi: https://doi.org/10.18488/journal.ajard.2021.111.1.9.

[14] Seleim, M. A., Abo-Elyousr, K. A., Abd-El-Moneem, K. M., and Saead, F. A. 2014. First Report of Bacterial Wilt Caused by *Ralstonia Solanacearum* Biovar 2 Race 1 on Tomato in Egypt. *The Plant Pathology Journal.* 30(3): 299
Doi: https://doi.org/10.5423/PPJ.NT.10.2013.0101.

[15] Lessler, J., Azman, A. S., McKay, H. S., & Moore, S. M. 2017. What is a Hotspot Anyway? *The American Journal of Tropical Medicine and Hygiene*. 96(6): 1270-1273.
Doi: https://doi.org/10.4269/ajtmh.16-0427.

[16] Campbell, C. L., & Madden, L. V. 1990. *Introduction to Plant Disease Epidemiology*. John Wiley & Sons.

[17] Groth, J. V., Ozmon, E. A., & Busch, R. H. 1999. Repeatability and Relationship of Incidence and Severity Measures of Scab of Wheat Caused by *Fusarium Graminearum* In Inoculated Nurseries. *Plant Disease*. 83(11): 1033-1038.
Doi: https://doi.org/10.1094/PDIS.1999.83.11.1033.

[18] Abd Samat, N. M. A., Ahmad, S., Awang, Y., Bakar, R. A. H., and Hakiman, M. 2020. Alterations in Herbage Yield, Antioxidant Activities, Phytochemical Contents, and Bioactive Compounds of Sabah Snake Grass (*Clinacanthus Nutans L.*) with Regards to Harvesting Age and Harvesting Frequency. *Molecules*. 25(12): 2833.
Doi: https://doi.org/10.3390/molecules25122833.

[19] Abu-Bakar, N., Juri, N. M., Abu-Bakar, R. A. H., Sohaime, M. Z., Badrun, R., Sarip, J., Hassan, M. A., & Ahmad, K. 2021. Recombinant Protein Foliar Application Activates Systemic Acquired Resistance and Increases Tolerance

against Papaya Dieback Disease. *Asian Journal of Agriculture and Rural Development*. 11(1): 1-9.
Doi: https://doi.org/10.18488/journal.ajard.2021.111.1.9.
[20] Waznul Adly M. Z., Alizah, Z., Maheswary, V. and Zuraida, A. R. 2010. Validation of Housekeeping Genes for Quantitative Real-time PCR in Malaysian Papaya var Eksotika I (*Carica papaya*). *Proceedings of UMS Biotechnology Symposium IV,* Kota Kinabalu, Sabah. Kota Kinabalu: UMS.
[21] Schmittgen, T. D., and Livak, K. J. 2008. Analyzing Real-time PCR Data by the Comparative CT Method. *Nature Protocols.* 3(6): 1101-1108.
Doi: https://doi.org/10.1038/nprot.2008.73.
[22] Bocsanczy, A. M., Nissinen, R. M., OH, C. S., and Beer, S. V. 2008. HrpN of *Erwinia Amylovora* Functions in the Translocation of DspA/E into Plant Cells. *Molecular Plant Pathology*. 9(4): 425-434.
Doi: https://doi.org/10.1111/j.1364-3703.2008.00471.x.
[23] Amil-Ruiz, F., Blanco-Portales, R., Munoz-Blanco, J., and Caballero, J. L. 2011. The Strawberry Plant Defense Mechanism: A Molecular Review. *Plant and Cell Physiology*. 52(11): 1873-1903.
Doi: https://doi.org/10.1093/pcp/pcr136.
[24] Sekeli, R., Hamid, M. H., Razak, R. A., Wee, C. Y., and Ong-Abdullah, J. 2018. Malaysian *Carica papaya L. var. Eksotika*: Current Research Strategies Fronting Challenges. *Frontier Plant Science*. 9: 1380.
Doi: https://doi.org/10.3389/fpls.2018.01380.
[25] Mohd Azhar, H., Mohd Zulfa., and Abdul Razak, M. 2020. Field Performance of Selected Papaya Hybrids for Tolerance to Dieback Disease. *Journal of Tropical Agricultureand Food Science*. 48(1).
[26] Liu, Y., Zhou, X., Liu, W. *et al*. 2020. HpaXpm, a Novel Harpin of *Xanthomonas phaseoli* pv. *manihotis*, Acts as an elicitor with High Thermal Stability, Reduces Disease, and Promotes Plant Growth. *BMC Microbiol*. 20(4). https://doi.org/10.1186/s12866-019-1691-4.
[27] Chen, L., Zhang, S. J., Zhang, S. S., Qu, S., Ren, X., Long, J., and Dong, H. 2008. A Fragment of the *Xanthomonas oryzae pv. oryzicola* Harpin HpaGXooc Reduces Disease and Increases Yield of Rice in Extensive Grower Plantings. *Phytopathology*. 98(7): 792-802.
Doi: https://doi.org/10.1094/PHYTO-98-7-0792.
[28] Maktar, N. H., Kamis, S., Mohd Yusof, F. Z., and Hussain, N. H. 2008. *Erwinia papayae* Causing Papaya Dieback in Malaysia. *Plant Pathology*. 57: 774.
DOI: https://doi.org/10.1111/j.1365-3059.2008.01877.x.
[29] Mat Amin, N., Bunawan, H., Redzuan, R. A., and Jaganath, I. B. S. 2011. *Erwinia mallotivora sp.,* A New Pathogen of Papaya (*Carica papaya*) in Peninsular Malaysia. *International Journal of Molecular Science*. 12: 39-45.
Doi: https://doi.org/10.3390/ijms12010039.
[30] Wang, D., Wang, B., Wang, J., Wang, S., Wang W., and Niu, Y. 2020. Exogenous Application of Harpin Protein Hpa1 onto *Pinellia ternata* Induces Systemic Resistance Against Tobacco Mosaic Virus. *Phytopathology*. 110: 1189-1198.
Doi: https://doi.org/10.1094/PHYTO-12-19-0463-R.
[31] Chakraborty, J., Ghosh, P., and Das, S. 2017. Autoimmunity in Plants. *Planta*. 248(4): 751-767.
Doi: https://doi.org/10.1007/s00425-018-2956-0.
[32] Cai, Z., Wang, Z., Yue, C., Sun, A., and Shen, Y. 2021. Efficient Expression and Purification of Soluble HarpinEa Protein by Translation Initiation Region Codon Optimization. *Protein Expression and Purification*. 188: 105970.
Doi: https://doi.org/10.1016/j.pep.2021.105970.
[33] Noor Shahida, Y., Awang, Y., Sijam, K., Noriha, M. A., and Satar, M. G. M. 2016. Biochemical Changes and Leaf Photosynthesis of *Erwinia mallotivora* Infected Papaya (*Carica papaya*) Seedlings. *American Journal of Plant Physiology*. 11: 12-22.
Doi: https://doi.org/10.3923/ajpp.2016.12.22.
[34] Fallahi, E. 2021. Harpin Protein Influence on Fruit Quality Attributes, Ethylene, Respiration, and Minerals in Apples. *American Journal of Plant Sciences*. 12(8): 1236-1245.
Doi: https://doi.org/10.4236/ajps.2021.128086.
[35] Kretschmer, M., Damoo, D., Djamei, A., and Kronstad, J. 2020. Chloroplasts and Plant Immunity: Where are the Fungal Effectors? *Pathogens.* 9(1): 19.
Doi: https://doi.org/10.3390/pathogens9010019.
[36] Yang, F., Xiao, K., Pan, H., & Liu, J. 2021. Chloroplast: The Emerging Battlefieldin Plant–Microbe Interactions. *Frontiers in Plant Science*. 12: 218.
Doi: https://doi.org/10.3389/fpls.2021.637853.
[37] Abd Elhady, S. I., & Abdulwahab, A. M. 2019. The Role of Green Energy as a Filter in Reviving the Industrial Spaces using the Green Microalgae Technology as a Landscape Feature (Rainbow of the Land)(A Practical Case Study of Industrial Area, German)-Article Review. *African Journal of Biological Sciences.* 15(1): 269-287.
Doi: https://doi.org/10.21608/ajbs.2019.194858.
[38] Zhao, B., Erwin, A., & Xue, B. 2018. How Many Differentially Expressed Genes: A Perspective from the Comparison of Genotypic and Phenotypic Distances. *Genomics*. 110(1): 67-73.
Doi: https://doi.org/10.1016/j.ygeno.2017.08.007.
[39] Diaz, I. 2018. Plant Defense Genes against Biotic Stresses. *International Journal of Molecular Sciences*. 19(8): 2446.
Doi: https://doi.org/10.3390/ijms19082446.
[40] Husaini, A. M., and Abdin, M. Z. 2008. Overexpression of Tobacco Osmotin Gene Leads to Salt Stress Tolerance in Strawberry (*Fragaria× ananassa Duch*.) Plants. *Indian Journal of Biotechnology*. 7: 465-471.
[41] Viktorova, J., Rehorova, K., Musilova, L., Suman, J., Lovecka, P., and Macek, T. 2019. New Findings in Potential Applications of Tobacco Osmotin. *Protein Expression and Purification*. 129: 84-93.
Doi: https://doi.org/10.1016/j.pep.2016.09.008.
[42] Bashir, M. A., Silvestri, C., Ahmad, T., Hafiz, I. A., Abbasi, N. A., Manzoor, A., and Rugini, E. 2020. Osmotin: A Cationic Protein Leads to Improve Biotic and Abiotic Stress Tolerance in Plants. *Plants.* 9(8): 992.
Doi: https://doi.org/10.3390/plants9080992.
[43] Alhaithloul, H. A., Soliman, M. H., Ameta, K. L., El-Esawi, M. A., and Elkelish, A. 2019. Changes in Ecophysiology, Osmolytes, and Secondary Metabolites of the Medicinal Plants of *Mentha piperita* and *Catharanthus roseus* Subjected to Drought and Heat Stress. *Biomolecules.* 10(1): 43.
Doi: https://doi.org/10.3390/biom10010043.
[44] Ullah, A., Hussain, A., Shaban, M., Khan, A. H., Alariqi, M., Gul, S., and Munis, M. F. H. 2018. Osmotin: A Plant Defense Tool against Biotic and Abiotic Stresses. *Plant Physiology and Biochemistry*. 123: 149-159.
Doi: https://doi.org/10.1016/j.plaphy.2017.12.012.
[45] Dana, M. D. L. M., Pintor-Toro, J. A., & Cubero, B. 2006. Transgenic Tobacco Plants Overexpressing Chitinases of Fungal Origin Show Enhanced Resistance to Biotic and Abiotic Stress Agents. *Plant Physiology*. 142(2): 722-730.
Doi: https://doi.org/10.1104/pp.106.086140.
[46] Datta, K., Velazhahan, R., Oliva, N., Ona, I., Mew, T., Khush, G. S., and Datta, S. K. 1999. Over-expression of the Cloned Rice Thaumatin-like Protein (PR-5) Gene in Transgenic Rice Plants Enhances Environmental Friendly Resistance to *Rhizoctonia solani* Causing Sheath Blight Disease. *Theoretical and Applied Genetics*. 98(6): 1138-1145.
[47] Sarowar, S., Kim, Y. J., Kim, E. N., Kim, K. D., Hwang, B. K., Islam, R., and Shin, J. S. 2005. Overexpression of a Pepper Basic Pathogenesis-related Protein 1 Gene in Tobacco Plants Enhances Resistance to Heavy Metal and Pathogen Stresses. *Plant Cell Reports.* 24(4): 216-224.
Doi: https://doi.org/10.1007/s00299-005-0928-x.
[48] Linthorst, H. J., and Van Loon, L. C. 2008. Pathogenesis-related Proteins of Plants. *Critical Reviews in Plant Sciences.* 10(2): 123-150.

Doi: https://doi.org/10.1080/07352689109382309.
[49] Cutt, J. R., & Klessig, D. F. 1992. Pathogenesis-related Proteins. In Genes Involved in Plant Defense. Springer. 209-243.
Doi: https://doi.org/10.1007/978-3-7091-6684-0_9.
[50] Ali, S., Ganai, B. A., Kamili, A. N., Bhat, A. A., Mir, Z. A., Bhat, J. A., and Grover, A. 2018. Pathogenesis-related Proteins and Peptides as Promising Tools for Engineering Plants with Multiple Stress Tolerance. *Microbiological Research*. 212: 29-37.
Doi: https://doi.org/10.1016/j.micres.2018.04.008.
[51] Dzhavakhiya, V. G., Ozeretskovskaya, O. L., and Zinovyeva, S. V. 2007. Immuneresponse. In Comprehensive and Molecular Phytopathology. Elsevier. 265-314.
[52] Durner, J., Shah, J., and Klessig, D. F. 1997. Salicylic Acid and Disease Resistance in Plants. *Trends in Plant Science*. 2(7): 266-274.
Doi: https://doi.org/10.1016/S1360-1385(97)86349-2x.
[53] Hu, Z., Zhang, H., and Shi, K. 2018. Plant Peptides in Plant Defense Responses. *Plant Signaling & Behavior*. 13(8).
Doi: https://doi.org/10.1080/15592324.2018.1475175.
[54] De León, I. P., and Montesano, M. 2013. Activation of Defense Mechanisms against Pathogens in Mosses and Flowering Plants. *International Journal of Molecular Sciences*. 14(2): 3178-3200.
Doi: https://doi.org/10.3390/ijms14023178.