# Enhancing Eksotika Papaya Resistance to Dieback Disease through Quorum Quenching

**Rogayah Sekeli*, Nazrul Hisham Nazaruddin, Amin Asyraf Tamizi, Noriha Mat Amin, Chien-Yeong Wee, Johari Sarip, Nora'ini Abdullah, Nurain Izzati Saidi, Roslinda Abdul Razak and Zaifulfarizal Zulkifli**

Biotechnology and Nanotechnology Research Centre, Malaysian Agricultural Research and Development Institute (MARDI), P.O. Box 12301, 50774 Kuala Lumpur, Wilayah Persekutuan

**Email: lynn@mardi.gov.my*

## ABSTRACT

The main objective of this study was to develop transgenic Malaysian Eksotika papaya with enhanced resistance to papaya dieback disease (PDD). Papaya dieback disease, which is caused by a Gram-negative bacterium, *Erwinia mallotivora*, has adversely affected Malaysia papaya industry and resulted in major production declines and economical losses. To rejuvenate the papaya industry as well as to improve papaya export, effective disease prevention strategies are imperative to curb the spread of the pathogen. Apart from the conventional breeding method, an alternative strategy by using genetic engineering approach was proposed to produce papaya plants that can silence quorum sensing system of the bacterial pathogen. Hence, by disrupting the communication mechanism, or known as quorum quenching, the disease development was hypothesised to be delayed or inhibited. An *acyl-homoserine lactone* (*AHL***)** *lactonase* gene isolated from rhizosphere soil bacteria strain CHB37 which has high quorum quenching potential against *E. mallotivora*, has been isolated, validated and transformed into embryogenic papaya calli. Out of 150 putative transgenic lines recovered, 70 lines were found positive with the presence of inserted transgenes. Screening of 3-month-old transgenic plants with *E. mallotivora* confirmed that *AHL lactonase* CHB37 gene has successfully delayed *E. mallotivora* infection in 12 transgenic papaya lines. The transgenic plants were able to survive and produce new shoot whereas control plants were mostly dead by the tenth-day post inoculation. It offers a promising outcome, nevertheless more screening will be conducted for further validation.

**Keywords**: Eksotika papaya, *Erwinia mallotivora*, papaya dieback disease, *AHL lactonase* gene

## INTRODUCTION

The papaya industry in Malaysia has been suffering from a serious papaya dieback disease (PDD) since 2003. This disease is capable of destroying the whole papaya plantation which eventually resulted in total production lost. Moreover, the disease can be spread to other papaya plantations in the vicinity through soil, air and other environmental factors (Figure 1).

One of the affected papaya cultivars was Eksotika papaya (*Carica papaya* L. cv. Eksotika), which has a high commercial value for export. Food and Agriculture Organisation of the United Nations Statistics Division **(**FAOSTAT) reported that papaya fruit production in Malaysia was reduced nearly 40% while the export value declined up to 70% from 2003 to 2011 (FAOSTAT, 2017). Papaya dieback disease was the actual reason behind these upsetting figures. The outbreak of PDD was first identified near Batu Pahat, Johor in late 2003 by the Johor State Department of Agriculture. The second outbreak was reported in Bidor, Perak, in October 2004. The outbreak affected 800 hectares of plantation and resulted in the destruction of approximately 1 million trees nationwide. The papaya industry subsequently collapsed with estimated total fruit yield loss around 200,000 metric tonnes, equivalent to USD 58 million (Maktar et al., 2008; Roshidi, 2010). Besides Eksotika, other cultivars affected by PDD were Solo, Hong Kong and Sekaki.

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Noriha et al. (2011) identified and confirmed the pathogen causing PDD is a Gram-negative bacterium, *E. mallotivora* from the family of Enterobacteriaceae. The use of pesticides and antibiotics were found ineffective in controlling the disease. Current strategy to control the disease is by having an effective management strategy, which includes early detection of symptoms development and removal of diseased papaya trees.. Affected trees will be chopped off and buried into the soil as recommended under the Plant Protection Act 1974.

(A) (B)

Figure 1. Symptoms of papaya dieback disease caused by *E. mallotivora*. (A) Water-soaked lesions formed on the stem and (B) leaf showing spots formed along the main vein (B).

To save our local papaya industry, especially Eksotika which has a great commercial value, an alternative strategy to combat this disease was conducted via genetic engineering approach. The focus of this research was to develop dieback-resistant Eksotika papaya by targeting bacterial quorum sensing mechanism. Quorum sensing is a communication process in Gram-negative bacteria which depends on cell population density. It is mediated by small signal molecules known as autoinducers which are produced and released by the bacterial cell into the extracellular environment where the concentration will increase in proportion to bacterial cell growth (Miller and Bassler, 2001). Low concentration of this signal molecule would not give negative effect to the plant. However, once the signal molecules exceed a threshold concentration, they will activate the virulence genes in *E. mallotivora* and subsequently cause PDD development in the papaya plant. Hence, by disrupting the essential communication mechanism through quorum quenching, the signal molecules will be degraded thus eliminating the bacterial virulence. Ultimately, this will reduce the infection of *E. mallotivora* on the papaya plants and consequently delaying or inhibiting the development of dieback disease.

It was reported that there are two types of enzymes capable of degrading the quorum sensing-signalling molecules, N-Acyl homoserine lactones (AHLs), and quench the quorum sensing behaviour known as lactonases and acylases (Rashid et al., 2011). The AHL lactonase, or termed as AiiA is responsible for hydrolysing one of the bonds in the lactone ring present in AHLs, thereby opening the ring and inactivating the AHLsignals (Dong et al., 2000). When the signal molecules in the surrounding environment do not reach the maximum threshold levels, the virulence genes that cause the disease will not be activated. Thus, a transgenic plant was hypothesised to be more resilient to the disease by harnessing the quorum quenching capability.

There have been a few success studies reported on the transformation of *AHL lactonase* gene from *Bacillus* species into crop plants such as tobacco, potato and elephant yam (Ban et al., 2009; Dong et al., 2000). In 2013, screening for potential genes involved in quorum quenching to be used in genetic manipulation studies against the dieback disease was initiated (Noriha et al., 2013). *AHL lactonase* gene used in this study was isolated from rhizosphere soil bacteria, *Bacillus cereus* strain CHB37, and was validated to have anti-quorum sensing activity against *E. mallotivora in vitro (*Noriha et al., 2013). The

DNA sequence of this *AHL lactonase* CHB37 gene showed 88 to 99% identity when compared with nucleotides of *AHL lactonase* from *Bacillus* species listed in the GenBank database. It was postulated that transgenic papaya plants expressing *AHL lactonase* is capable of quenching the pathogen quorum-sensing signalling and enhancing their defence againstPDD. Hence, this gene was selected for *Agrobacterium*-mediated transformation of embryogenic calli of Eksotika papaya for trait improvement.

## MATERIALS AND METHODS

### *Acyl-homoserine lactonase* gene cassette development

Full length *AHL lactonase* gene CHB37 with the size of 753 bp was isolated from *Bacillus cereus* strain CHB37 and cloned into plant transformation vector, pCAMBIA 2301. The *AHL lactonase* gene was inserted in sense orientation into pCAMBIA 2301 plasmid at *gus* site. The pCAMBIA 2301 vector contained the selectable marker gene, *neomycin phosphotransferase* (*npt*II) which was conferring resistance to antibiotic kanamycin and the inserted gene was driven by a cauliflower mosaic virus 35S promoter and nos terminator. The gene cassette constructed was validated using polymerase chain reaction (PCR), restriction enzyme digestion and DNA sequence analysis. This sense gene cassette was designated as pCAhl-CHB37. The gene cassette which gave the correct insert size with the right orientation was transformed into *Agrobacterium tumefaciens* strain LBA4404 using electroporation method and used for *Agrobacterium*-mediated transformation of one-month old embryogenic calli.

### Embryogenic callus induction and *Agrobacterium*-mediated transformation

Embryogenic callus was induced from immature zygotic embryo cultured on a callus induction medium which consisted of half-strength Murashige and Skoog medium (Murashige and Skoog, 1962), supplemented with 50 mg/L myo-inositol, full-strength MS vitamin, 6% (w/v) sucrose, 45.2 µM 2,4-dichlorophenoxyacetic acid and 0.35% (w/v) phytagel. The pH of the media was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. The cultures were grown at 25 ± 2°C in the dark for one month.

One-month-old embryogenic calli produced were transformed with the constructed gene cassette using a previously established *Agrobacterium*-mediated transformation method for Eksotika (Vilasini et al., 2000). Embryogenic calli were transformed with the *AHL lactonase* gene cassette and empty pCAMBIA 2301 vector (without gene of interest) as the control. The selection of putative transformed tissue was carried out for a total of four months on half-strength MS medium supplemented with kanamycin. Selection process was started with lower concentration of kanamycin at 75 mg/L for one month and then followed by 150 mg/L kanamycin for the remaining 3 months.

### Plant regeneration of putative transformed tissues

The survived putative transgenic calli on the selection media were transferred to the MS maturation medium without plant hormone for further development of somatic embryos. After 1 month, the somatic embryos that developed on the maturation medium were placed onto De Fossard regeneration medium (De Fossard et al., 1974) supplemented with 0.89 µM benzylaminopurine (BAP) and 1.1 µM 1-napthaleneacetic acids (NAA) to form plantlets. The regenerated plantlets which reached 3 to 4 cm in height were transferred into rooting media following previously established method (Rogayah et al., 2013) and the rooted plants were transferred into soil in polybag. All the cultures were grown at 25 ± 2°C in light condition.

### Polymerase chain reaction (PCR) analysis of putative transgenic papaya plants

The presence of transgenes in putative transgenic papaya plants was evaluated using PCR analysis. Genomic DNA was extracted from the leaves of putative transgenic plants of approximately 8 to 10

months old using the Qiagen kit (Qiagen, Germany) with a starting material of 100 mg for each sample. Approximately 50 ng of each extracted genomic DNA sample was subjected to PCR analysis using the specific primer for *AHL lactonase* gene (pCAhl-CHB37 forward 5'-ccatggatgacagtaaag-3' and pCAhl-CHB37 reverse 5'- ggtcacctatatattccgg -3'), as well as a primer pair for *npt*II gene (*npt*II forward 5'-ccttatccgcaacttctttacc-3' and *npt*II reverse 5'-caccatgatattcggcaagcag-3'). The reaction was carried out according to the PCR conditions which consisted of initial denaturation step for 3 min at 94°C followed by 35 cycles of 94°C for 1 min, 50°C for 45 sec, 72°C for 1 min and a final elongation step at 72°C for 10 min.

### Evaluation of transgenic papaya plants against *E. mallotivora*

Independent transgenic Eksotika papaya lines produced were grown in a control environment in Transgenic Glasshouse Complex condition for evaluation of resistance against *E. mallotivora*. Standard fertilisation and pest control programs were applied for plant maintenance. Three-month-old plants were used for screening whereby the plants were inoculated with *E. mallotivora* by prick-and-spraying method. Seedling-derived non-transformed papaya plants at 3 months old were used as positive controls to confirm symptom development after inoculation. Before spraying, all 70 independent transgenic papaya lines and control plants were injected with sterile distilled water at three different points to induce wounding. Then, 5 mL overnight culture of *E. mallotivora* at a concentration of $10^6$ colony-forming units (cfu) and an absorbance reading of 0.5 at $A_{600}$ were used for spraying. Symptom developments were observed and recorded daily.

## RESULTS AND DISCUSSION

### *Agrobacterium*-mediated transformation and generation of transgenic papaya plants

The *AHL lactonase* CHB37 gene with high quorum quenching potential against *E. mallotivora* was isolated from rhizosphere soil bacterium of highland tomato in Malaysia and identified as *Bacillus cereus*. The nucleotide sequence of this *AHL lactonase* CHB37 contained an open reading frame (ORF) of 753 bp nucleotide which encodes a polypeptide of 251 amino acids (Figure 2). Previous study indicated that this gene has anti-quorum sensing and antagonism activity against papaya dieback pathogen, *E. mallotivora* and therefore it can be utilised in genetic transformation of Eksotika papaya targeting dieback resistance (Noriha et al., 2013).

For gene cassette development, the *AHL lactonase* gene was inserted in sense orientation into the pCAMBIA 2301 plasmid at *gus* site. The gene cassette contains a cauliflower mosaic virus (CaMV) 35S promoter and nos terminator. Strong constitutive promoter, CaMV 35S, was selected in this study to control the expression of *AHL lactonase* gene. Figure 3 showed the PCR analysis for gene cassette pCAhl_CHB37. PCR results showed that *AHL lactonase* gene was successfully cloned into pCAMBIA 2301. DNA sequence results also confirmed the inserted *AHL lactonase* are in the right orientation in the constructed gene cassette. The *AHL lactonase gene* cassette was successfully electroporated into *A. tumefaciens* strain LBA4404 and introduced into Eksotika papaya embryogenic calli via *Agrobacterium*-mediated transformation. The empty pCAMBIA 2301 was also transformed into competent *A. tumefaciens* strain LBA4404 and used as a transformation controls besides non-transformed tissue culture control.

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  1 ATGACAGTAA AGAAGCTTTA TTTCGTGCCA GCTGGTCGTT GTATGTTAGA TCATTCTTCT
 61 GTTAATAGTA CGCTCACACC GGGGAATTTA TTAAACTTAC CAGTATGGTG TTATTTATTG
121 GAGACAGAAG AAGGGCCAAT TTTAGTAGAT ACAGGTATGC CAGAAAGTGC GGTTAATAAT
181 GAAAATTTAT TTGACGGTAC ATTTGTTGAA GGGCAGATTT TACCGAAAAT GACTGAAGAA
241 GATAGGATCG TAAATATTTT AAAACGCGTT GGTTATGAGC CGGAAGACCT TCTTTATATT
301 ATTAGTTCTC ACTTGCATTT TGATCATGCA GGGGGAAATG GTACTTTTAC AAATACACCG
361 ATTCTTGTGC AGCGTGCTGA ATATGAGACG GCACAACATA GTGAAGAATA TTTGAAAGAA
421 TGTATATTGC CGAATTTAAA CTACAAAATC ATTGAAGGGG ATTATGAAGT CGTACCAGGT
481 GTTCAATTAT TGTATACACC AGGACATACT CCAGGACATC AGTCACTATT CATTGAGACG
541 GAAAACTCTG GCCCAGTGTT ATTAACGATC GATGCATCGT ATACGAAAGA GAATTTTGAA
601 GATGAAGTGC CGTTCGCGGG GGTTGATTCG GAATTAGCTT TATCTTCAAT TAAGCGTTTA
661 AAAGAAATTG TTAGGAAAGA GAACCCAATT GTTTTCTTTG GACATGATAT AGAACAGGAA
721 AAGAGCTGTA AAGTGTTCCC GGAATATATA TAG
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Figure 2. Nucleotide sequence of *AHL lactonase* CHB37 gene

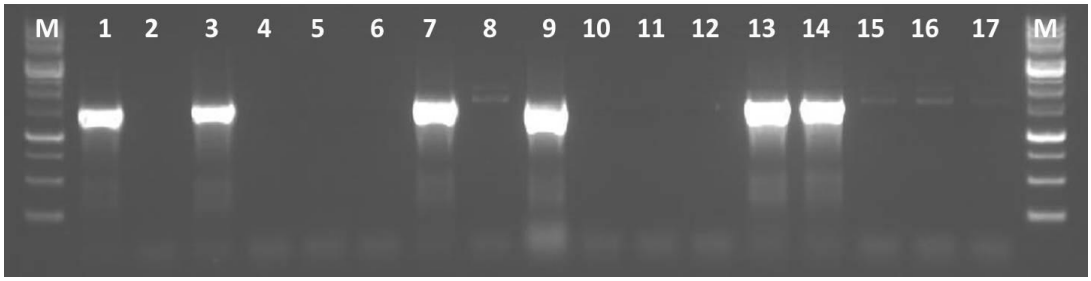


Figure 3. Colony PCR of pCAhl-CHB37 recombinant plasmids using the forward primer located at the 35S promoter (laz_F) and reverse primer at the *AHL lactonase* (~1.4 kb). PCR results showed that *AHL lactonase* gene was successfully cloned into pCAMBIA 2301 (lanes 1, 3, 7, 9, 13 and 14). Lane M represented 1 kb DNA Ladder as molecular weight markers.

Embryogenic calli induced from immature zygotic embryo of Eksotika papaya were used as the explants for *Agrobacterium*-mediated transformation. A total of 4,000 embryogenic calli of Eksotika papaya were transformed with the *AHL lactonase* gene cassette and 600 were transformed with vector control (pCAMBIA 2301 without *AHL lactonase* gene). After 4 months selection on kanamycin medium, a total of 150 putative transformed calli were recovered. The putative transformed calli that survived on the selection medium were sub-cultured onto maturation medium without any plant growth regulators to enhance the formation of somatic embryos and to reduce the incident of abnormal plants due to stress during the selection process. Then the somatic embryos were transferred onto regeneration De Fossard medium supplemented with BAP and NAA to form plantlets. More than 90% of the putative transformed calli were successfully regenerated into normal plantlets after 6 to 8 months culture on the regeneration medium (Figure 4).

In addition, two different negative controls were set up for this transformation experiment. The first control was embryogenic calli transformed with empty vector. The second control was tissue culture control whereby the one-month old embryogenic calli were directly transferred onto the maturation and regeneration media without selection process on kanamycin medium. While the transformed vector control calli went through the selection process on kanamycin medium for 4 months similar with the gene transformed calli to identify the putative transformed tissues. Finally after the final selection process, 46 putative transformed calli were successfully recovered.

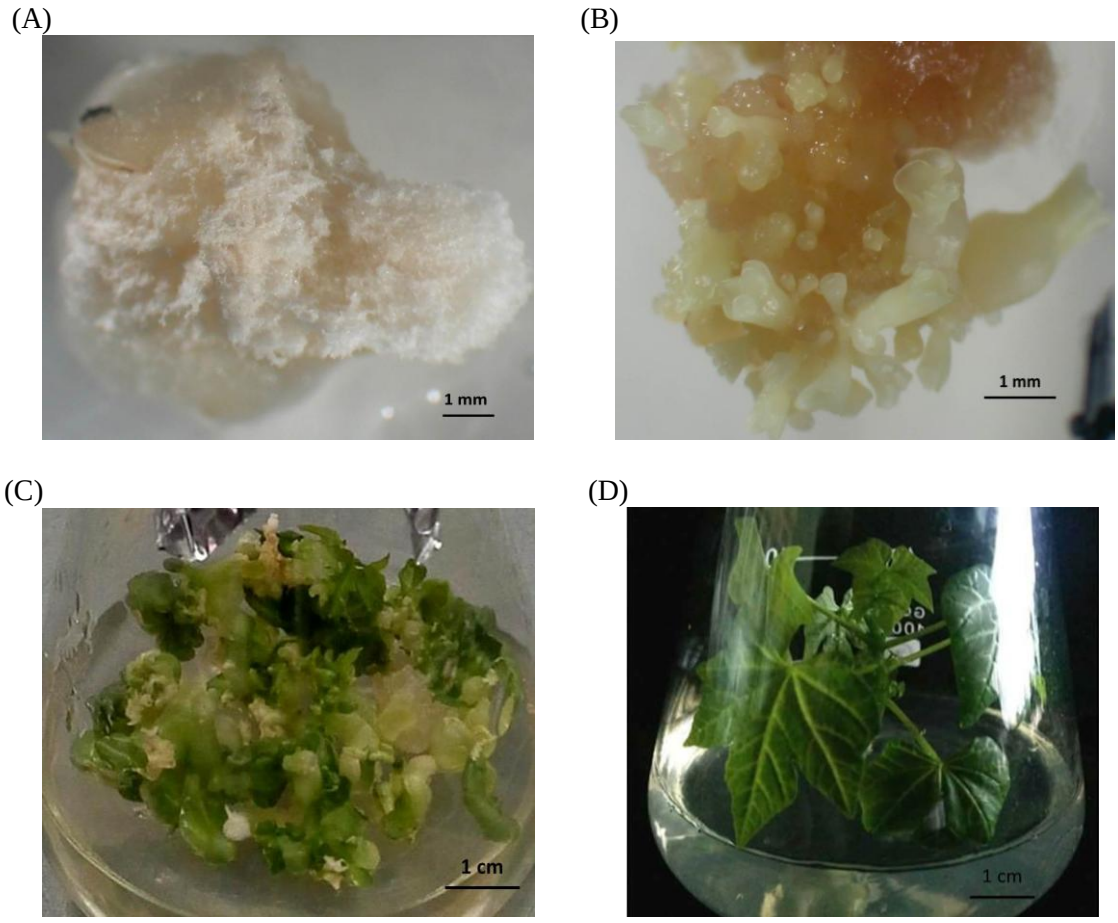


Figure 4. *Agrobacterium*-mediated transformation of Eksotika papaya. (A) 1-month old embryogenic callus; (B) putative transformed calli that had survived on selection media; (C) putative transformed tissue, and (D) regenerated putative transformed plantlet.

### Molecular characterisation of generated $T_0$ putative transgenic papaya plants

To verify the integration of transgenes in the genome of putative transgenic papaya plants which were designated as transformed $T_0$ lines, PCR analysis was carried out on young leaves of regenerated putative transformed plants (Figure 5). The pCAMBIA plasmid containing *AHL lactonase* CHB37 gene was used as positive control while the genomic DNA from non-transformed calli was used as negative control to evaluate the PCR results. From a total of 150 independent lines analysed, 70 were positive for the presence of both *AHL lactonase* CHB37 and *npt*II genes. The results showed that *AHL lactonase* and *npt*II genes were successfully integrated into plant genome indicated by PCR fragments with the sizes of ~753 bp and ~800 bp, respectively. Overall, the percentage of positive transgenic papaya lines obtained was 1.75% based on the total number of calli transformed.

Meanwhile for vector transformed control plantlets, 23 (3.8%) were positive for the presence of *npt*II gene. The shoot of PCR positive transgenic plants were excised from shoot clusters and transferred to half-strength MS basal medium for 4 weeks prior to use in rooting experiment. Vermiculite supplemented with half strength MS was used as a substrate in rooting of positive transgenic papaya plants. The successful rooted transgenic plants were transferred into soil in polybag and acclimatised at the Transgenic Glasshouse Complex for further hardening before being challenged with *E. mallotivora*.

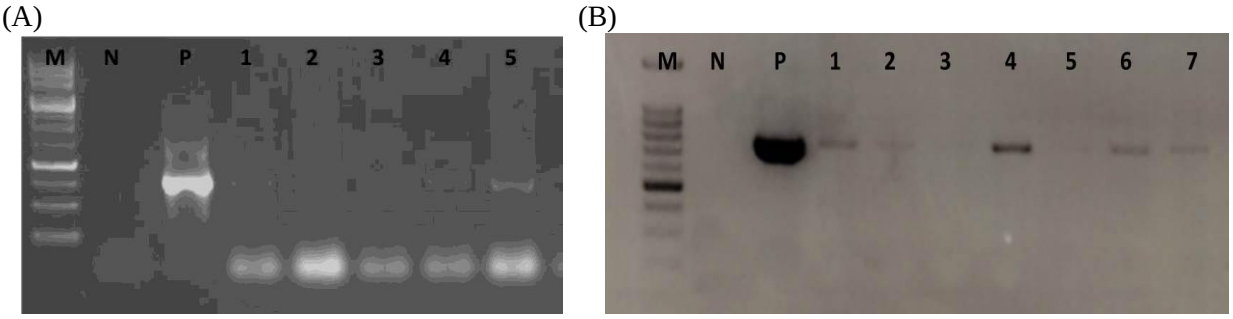


Figure 5. PCR analysis of putative transgenic papaya plants transformed with pCAhl-CHB37 construct. (A) PCR analysis results of putative transgenic lines (lanes 1 to 5) using specific primer for the *AHL lactonase* gene (~753 bp). Lane M: 1 kb DNA ladder, Lane N: negative control and Lane P: positive control (pCAMBIA 2301:*AHL lactonase* plasmid) and (B) PCR analysis results of putative transgenic lines (lanes 1 to 7) using *npt*II primer and the expected size is ~800 bp. Lane M: 100 bp DNA ladder, Lane N: negative control and Lane P: positive control (pCAMBIA 2301:*AHL lactonase* plasmid).

**Evaluation of dieback resistance in $T_0$ transgenic papaya lines**

The transgenic plants harbouring the anti-pathogenic *AHL lactonase* gene were further challenged with the causal agent of papaya dieback disease, *E. mallotivora*. A total of 70 independent transgenic papaya lines and 10 vector transformed plants were challenged against *E. mallotivora* to determine the degree of resistance against dieback disease. Spraying bacterial suspension at the wound site on the three-months-old plants was used as a method of inoculation with the purpose to mimic the environment condition and the experiments were carried out in Transgenic Glasshouse Complex condition. According to Noriha et al. (2011), *E. mallotivora* attacks the plants through open wounds, colonises and moves through the plant vascular system. The seedlings derived from non-transformed papaya plants were used as a positive control to validate symptoms development after inoculation. At this stage of screening, no replication was involved as all transgenic plants screened were considered as an independent line.

In seedling-derived non-transformed plants, the symptoms were observed after 3 days of inoculation but were delayed to 10 days in 12 transgenic papaya lines. Seedling-derived non-transformed papaya plants showed yellow mottling, distortion of young leaves and water-soaked lesions after 3 days of inoculation. On day 6, the severity of dieback symptoms on the control papaya plants could be seen clearly, whereby the leaves turned brown and some of the plants shed off their leaves. Due to water-soaked lesions, fungal infection was also observed. All the control plants died on the 10th day post-inoculation (Figure 6).

However, for $T_0$ transgenic papaya plants harbouring *AHL lactonase* gene, they did not exhibit dieback symptoms such as water soaked areas and brown spots on the same day. Even though the symptoms of the dieback disease were observed on day 10, the plants still survived for more than 30 days in 12 transgenic papaya lines and produced new shoots. In these transgenic papaya lines, the plants displayed symptoms only at the bacterial inoculation site without any further spreading and survival was observed past 30 days post-inoculation with formation of new shoots. The dieback disease symptoms on the rest of the transgenic papaya plants were observed up to 8 to 9 days post-infection. However, the plants failed to survive more than 20 days.

As reported by Dong et al. (2000), the cloning of *aiiA* gene encoding an *AHL lactonase* isolated from the *Bacillus* sp. strain 240B1 had successfully inactivated an AHL signalling in *Erwinia* sp. via hydrolysis of its lactone bond. The expression of this gene had enhanced the pectolytic enzyme activities against the *E. carotovora*, a causal agent of soft rots in many plants. In addition, the transgenic plants harbouring the *AHL lactonase* gene were reported to be resistant to *E. carotovora* infection and the soft rot

symptoms development were delayed (Dong et al., 2001). Similar results were obtained in this study whereby it was demonstrated that the expression of *AHL lactonase* CHB37 gene was capable of quenching the pathogen quorum-sensing signaling and it was proven by low pathogenic and slower spread of symptoms to other parts of the plant. Thus, a few of the transgenic lines were able to survive more than 30 days and produced new shoots. The interference of the quorum sensing system by the quorum quenching enzyme was an alternative strategy for traditional antibiotics method because the quorum quenching strategy does not aim to kill the pathogen or limit cell growth but to shut down the expression of the pathogenic gene. The shoot tips and axillary buds from 12 potential transgenic resistance papaya lines were isolated and mass propagated using tissue culture technique for further contained field evaluation in the near future.

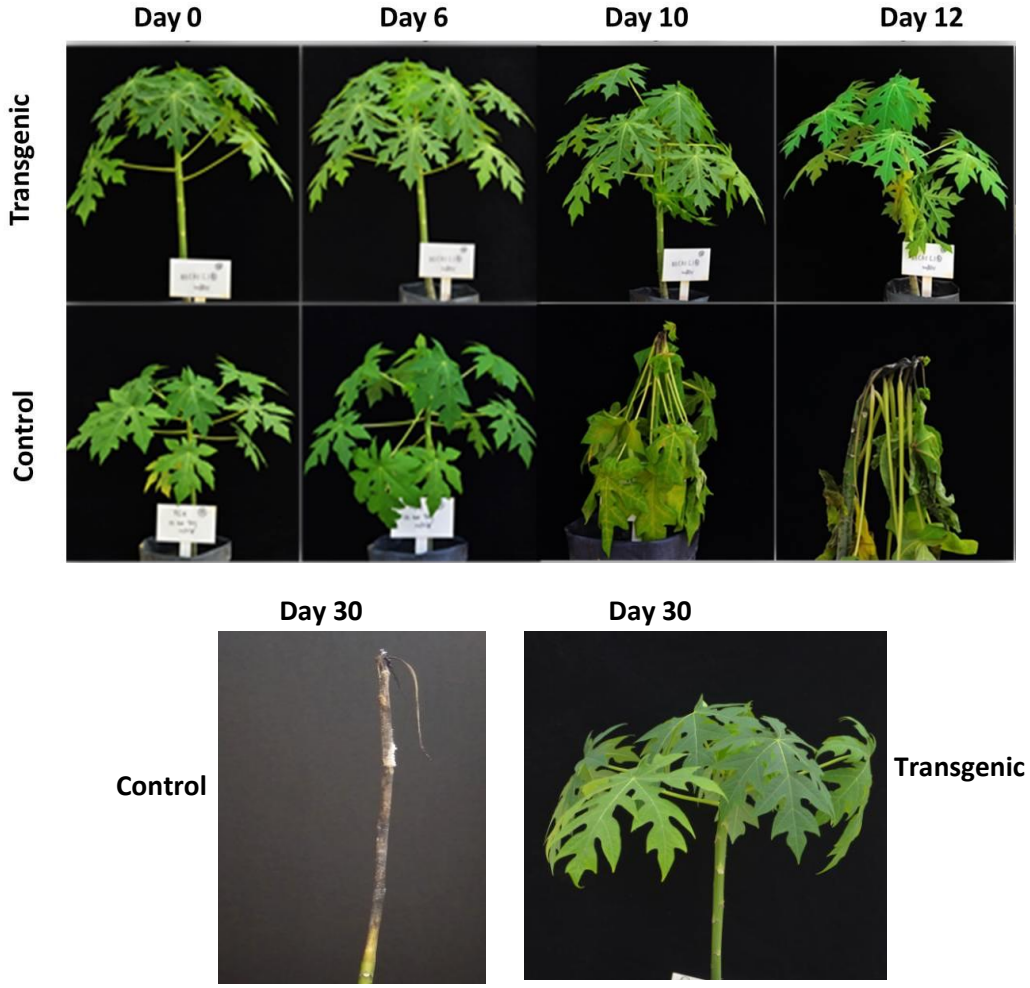


Figure 6. Performance of transgenic vs non-transgenic papaya plants during *E. mallotivora* infection.

## CONCLUSIONS

Our initial evaluation indicated that 12 transgenic Eksotika papaya lines transformed with *AHL lactonase* gene showed substantial delayed infection and mild symptom development after inoculation with *E. mallotivora.* These results suggested that transforming *AHL lactonase* gene into Eksotika papaya was indeed a promising approach to acquire resistance against PDD. However, further evaluations in

contained environment and molecular gene expression analysis are needed to be carried out for identification of the most potential lines to be used for subsequent confined and open field trials.

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