Australian Journal of Crop Science AJCS

AJCS 13(01):55-60 (2019) ISSN:1835-2707
doi: 10.21475/ajcs.19.13.01.p1169

Foliar application of chitosan enhances growth and modulates expression of defense genes in chilli pepper (*Capsicum annuum* L.)

Rizkita Rachmi Esyanti*, Fenny Martha Dwivany, Soraya Mahani, Husna Nugrahapraja and Karlia Meitha

School of Life Sciences and Technology, Institut Teknologi Bandung, West Java, Indonesia

***Corresponding author: rizkita@sith.itb.ac.id**

Abstract

Phytophthora capsici is one of the primary pathogens causing a global problem of severe losses in chilli production. The use of conventional fertilisers and fungicides to improve chilli production had been shown to elevate environmental and health issues. Hence, the foliar application of chitosan, natural deacylated chitin, to enhance growth and resistance in chilli pepper plants was investigated. The chilli plants were grown for 14 days before receiving chitosan application and 33 days before Phytophthora infection, physiological parameters were recorded during the growth period, and expression of resistance related genes was quantified at 72 hours after infection. Our results showed that physiological parameters, such as increment of height and leaves number, and chlorophyll content indicated an improved growth process in chitosan treated plants compared to the control. Plant resistance to Phytophthora infection was also investigated following chitosan application to highly (CM334), moderately (LABA), weakly (LADO) resistant and susceptible (15080) cultivars. The disease incidence and severity indices were reduced in chitosan-treated plants, except in highly and moderately resistant cultivars. Further, expression was also quantified for defence-related genes, including 9-lipoxygenase (*CaLOX*), Ca2+-bound calmodulin 1 (*CaCaM1*), receptor-like cytoplasmic protein kinase (*CaPIK*), Pto-interacting1 (*CaPTI1*) and resistance gene analogue 2 (*CaRGA2*). The results suggest that *CaLOX*, *CaPTI1* and *CaRGA2* genes were involved in defence mechanism to Phytophthora, with increased expression during infection. However, expression levels were reduced when Phytophthora infection was coupled with foliar chitosan application, indicating that chitosan may play a direct role in decreasing the pathogenicity of Phytophthora. In conclusion, this study suggests the promising role of chitosan as an alternative to conventional fertiliser and fungicide in chilli pepper plant.

Keywords: *Phytophthora capsici*, chitosan, *CaLOX*, *CaPTI1*, and *CaRGA2*.
Abbreviations: (*CaLOX*)_9-lipoxygenase; (*CaCaM1*)_Ca2+-bound calmodulin 1; (*CaPIK*)receptor-like cytoplasmic protein kinase, (CaPTI1)_Pto-interacting1, (CaRGA2)_resistance gene analogue 2.

Introduction

Chilli and chilli pepper (*Capsicum annuum* L.) are essential culinary herbs throughout the world. They are sought for the hot sensation and special flavour characters infused with food dishes. Global chilli and chilli pepper gross production value accounted for over USD 32 million in 2013, which represented 10% of total crop value worldwide. The total harvested area in the same year spanned up to 5.5 million hectares, producing >33 million tonnes of dry and green chillies (FAO, 2013). The fruit is also considered as a source of many nutrients, such as vitamin A and C, essential oil, antioxidants, capsaicin, flavonoids, steroid saponin, and capsicol (Prabaningrum and Moekasan, 2011).

Chilli pepper is one of the most economically valuable crops in Indonesia, a country that is famous for its spicy foods. Chilli production in Indonesia, from 2011 to 2015, was ranked as the fourth most abundantly grown produce after cabbage, potato and onion. However, domestic production of this herb does not meet the high demand of the country, pushing the monthly import quota to 1,600-3,500 tonnes in 2015 (Ministry of Agriculture, 2015). This mismatch between domestic supply and demand, according to Duriat et al. (2007), is due to the inability of driving chilli production to reach the maximum yield potential at 12-20 tonnes per hectare. This problem is attributed to severe yield losses caused by a cocktail of pathogens, with Phytophthora blight disease recognised as one of the most debilitating. Efforts to boost production include increasing fertiliser application. However, this has delivered no significant beneficial outcome to date. Further, the risks of land and water pollution due to excessive use of fungicide and fertiliser pose potential harms to the environment.

Chitosan is a form of deacetylation of chitin (a linear polysaccharide) which is abundant (Aranaz et al., 2010). Chitosan has unique biological and commercial properties: (a) a defined chemical structure, (b) the potential to be chemically and enzymatically modified, (c) physical and biological functionality, (d) biodegradability and biocompatibility with many organs, tissues, and cells, and (e) the potential to be processed into several products to facilitate applications (Harish and Tharanathan 2007). The chitosan polysaccharide is considered as a plant growth regulator (PGR) that was shown to increase the yield of okra

(Mondal et al., 2012) and cowpea (El-Tanahy et al., 2012), to stimulate growth in tissue culture (Nge et al., 2006) and to enlarge chloroplast of Dendrobium orchid (Limpanavech et al., 2008). Moreover, chitosan application as a PGR also offers convenience and low costs and labour efficiency (Mondal et al., 2012).

The role of chitosan as a potent fungicide in chili pepper has also been suggested by several studies in the literature (Taengchompoo et al., 2011; Long et al, 2017; Kewsuwan et al., 2014), each of which point to the disruptive interaction of chitosan with cell components of pathogens (Malerba and Cerana, 2016) as a major factor in plant survival. Several studies have also shown that survival of fungi-infected chilli pepper plants also relies upon the timely expression of defence related genes, such as 9-lipoxygenase (*CaLOX*), Ca2+-bound calmodulin 1 (*CaCaM1*), receptor-like cytoplasmic protein kinase (*CaPIK*), Pto-interacting1 (*CaPTI1*) and resistance gene analogue 2 (*CaRGA2*). *CaLOX* (Hwang and Hwang, 2010) and *CaCaM1* (Choi et al., 2009) are known to be involved in oxidative processes as a response to pathogen infection. Meanwhile, *CaPIK* (Choi et al., 2009) and *CaPTI1* (Jin et al., 2016) contribute to plant defence signalling and the regulation of resistance gene expressions, respectively. Lastly, silencing of *CaRGA2* expression in chilli pepper has significantly reduced the resistance of Phytophthora-infected plants (Zhang et al., 2013).

This presented study examines the effect of foliar application of chitosan to growth in chilli pepper plants (*Capsicum annuum* L.) as an alternative to conventional fertilisers and fungicide. Physiological and molecular approaches were performed to assess the effects of chitosan application. Growth parameters were quantified physiologically, whereas defence mechanism was measured through visible signs of infection and coupled with expression analysis of defence genes.

Results

Chitosan effects on physiological characters of growth in chilli pepper plants

In general, chitosan treated plants were healthier and grew taller than the control that was sprayed only with water (Fig. 1A). Results showed that the height of the highly resistant cultivar (CM334) significantly increased when treated with all chitosan concentrations. The commercial moderately LABA and weakly resistant LADO grew taller after treatments of 600 and 200 μg mL^{-1} chitosan, respectively. Similar to LADO, the tallest plants were observed after applying 200 μg mL^{-1} chitosan to the susceptible cultivar (15080).

Chlorophyll content in the leaves was highest in the susceptible cultivar (15080) when 200 200 μg mL^{-1} chitosan was applied. All concentrations of chitosan application resulted in higher chlorophyll contents in the weakly resistant LADO, but insignificant differences in the moderately resistant LABA. Chlorophyll content in the highly resistant CM334 was highest with chitosan application at 600 μg mL^{-1}. Figure 1C shows that there is no significant difference in leaf number of LABA and LADO cultivars between control and chitosan treated plants. Interestingly, in both highly resistant and susceptible cultivars plants treated with 1000 μg mL^{-1} chitosan produced more leaves.

Reduction of disease incidence and severity indices following chitosan application

The reduction of disease incidence and severity indices in Phytophthora-infected plants are shown in figure 1D and E. The data were recorded following foliar application of chitosan at concentrations of 0, 200, 600 and 1000 μg mL^{-1}. Disease incidence and severity indices were lower in chitosan treated plants of LADO (weakly resistant) and 15080 (susceptible) cultivars and were most significant when plants were sprayed with 1000 μg mL^{-1} chitosan. However, none of the chitosan treatments affected CM334 (highly resistant) and LABA (moderately resistant) cultivars following infection with 5.10^{4} cells of Phytophthora capsici zoospores.

Modulation of defence genes during Phytophthora infection and chitosan treatment

A set of five genes (*CaCAM1, CaPTI, CaPIK, CaLOX, and CaRGA2*) were selected based on the level of expression and modulation during pathogen infection in Capsicum annuum L. (Choi et al., 2009; Hwang and Hwang, 2010; Jin et al., 2016; Zhang et al., 2013; Kim and Hwang, 2011). To obtain the most contrasting results, only the susceptible (15080) and highly resistant (CM334) cultivars were selected for this analysis. Our results showed that the expressions of the five genes were modulated (Fig. 2). The *CaLOX* expression steeply increased in the Phytophthora infected susceptible cultivar (15080.PC), moderately increased when the Phytophthora was coupled with chitosan treatment (15080.PC-CHI) and was relatively low in chitosan treated only plants (15080.CHI). Interestingly, increased *CaLOX* expression was not recorded in the highly resistant cultivar, suggesting cultivar-specific modulation. CaCAM1 was highly expressed in CM334.CHI sample, but did not show any patterns of expression with other treatments. Similar results were also observed for *CaLOX*, *CaPTI1* and *CaRGA2*, each of which demonstrated high modulations during infection in the susceptible cultivar (15080.PC) but which were relatively lower when infection was coupled with chitosan treatment (15080.PC-CHI). The *CaPIK* expression seemed unresponsive to all tested treatments.

Discussion

Application of chitosan as fertiliser has been demonstrated to enhance growth and yield of okra (Mondal et al., 2012), cowpea (El-Tanahy et al., 2012) and mungbean (Rabbi et al., 2016). We also recorded an enhanced growth in chilli pepper plants following foliar application of chitosan, although yield was not one of the measured parameters. Plant height was significantly enhanced in all studied cultivars after chitosan application at specific concentrations. The same was also found for growth regarding leaf number except moderately and weakly resistant, LABA and LADO, respectively. Guixian et al. (2003) showed that application of N-carboxymethyl chitosan to maize resulted in elevated amounts of N and N protein as well as the activity of critical enzymes in N metabolism. Nitrogen is widely known as a nutrient required for chlorophyll, cytokinin, and various enzymes synthesis.

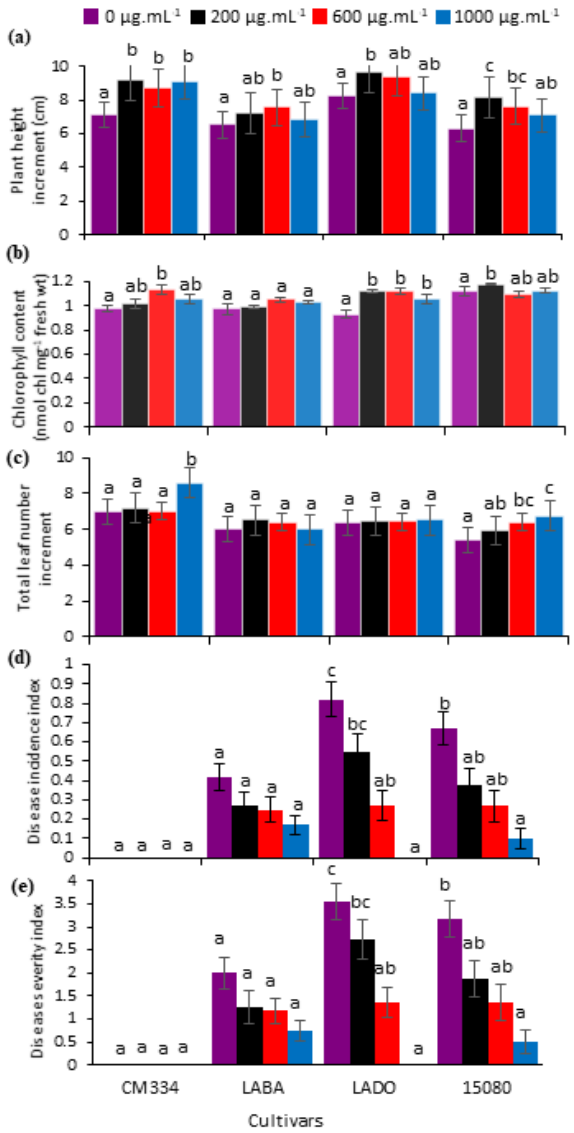

Fig 1. The physiological analysis of chilli pepper plant (Capsicum annuum L.) in response to chitosan application (a-c) and Phytophthora capsici infection (d-e). The analysis was made to highly (CM334), moderately (LABA), weakly (LADO) resistant and susceptible (15080) cultivars. Foliar feeding technique was applied by spraying chitosan solution at corresponding concentration, starting from day 14 post planting up to day 33. Measured parameters include increment in plant height (a); increment in leaves number (b); chlorophyll content (c); disease incidence (d) and severity (e) indices. Statistical significance is represented by the alphabet on top of each bar.

An increased capacity for photosynthesis might be one of the primary drives behind the enhanced growth of chitosan-treated plants. According to Khan et al., (2002), application of chitosan pentamer has significantly increased photosynthetic rate in soybean and maize leaves after a decrease in day one and two post-application. We also found that chlorophyll content in CM334, LADO and 15080 cultivars increased when treated with specific concentrations of chitosan. Similarly, in maize, application of Cu-chitosan nanoparticle increased chlorophyll concentration and plant growth in general (Choudhary et al., 2017). Furthermore, the chloroplast is reported to be one of the major sites for chitosan action in Dendrobium Eiskul, as chloroplasts were enlarged after long-term chitosan use (Limpanavech et al., 2008).

Systematic reviews on chitosan as a potent antimicrobial highlight the complex interactions between the active compound (chitosan) and target (microbes), and the capacity of chitosan to act as a plant elicitor, particularly in agriculture (Kong et al., 2010; Xing et al., 2015). Our study also showed that chitosan application had reduced both disease incidence (DII) and severity indices (DSI) in Phytophthora-infected chilli pepper plants. These results suggest that chitosan plays an inhibiting role when interacting with Phytophthora spores and limits further growth of the pathogen. While there are plenty of proposed methods of anti-microbial action of chitosan, the majority of them were investigated in bacteria (Malerba and Cerana, 2016). Research on chitosan action on Rhizopus stolonifera showed that direct interaction of chitosan with the negatively charged components of the cell surface is potentially the primary cause of inhibition of H^+-ATPase activity and chemiosmotic-driven transport (García-Rincón et al., 2010). Additionally, chitosan's role as a fungicide is primarily due to its action as plant elicitor to synthesise chitinase and β-1,3-glucanase (Xing et al., 2015). Both enzymes are known to act synergistically in the degradation of chitin-glucan of fungal cell walls. This capacity to produce specific hydrolytic enzymes in plants with no corresponding internal substrates may have been retained throughout evolution as a strategy to confront attacks by insect and fungi (Hadwiger, 2013).

As an exogenous elicitor, chitosan can induce the resistance of the host plant by modifying gene expression to promote defence mechanisms. Thus, we also investigated the modulation of defense-related gene expressions following Phytophthora infection and chitosan treatment in susceptible 15080 and highly resistant CM334 cultivars. We found that *CaLOX* expression was only modulated in the susceptible 16080 and was non-responsive in the resistant CM334, suggesting cultivar specific gene expression patterns. The *CaLOX* is responsible for peroxidation processes during plant-disease interaction and its silencing results in increased susceptibility of chilli pepper plants to Xanthomonas campestris pv vesicatoria (Xcv) and Colletotrichum coccodes (Hwang and Hwang, 2010). Interestingly, in the susceptible cultivar, chitosan treatment to Phytophthora-infected plants (CHI-PC) reduced *CaLOX* expression relative to plants without chitosan treatment (PC), while application to plants without Phytophthora infection (CHI) increased *CaLOX* expression relatively to control (CTRL). This study suggests that *CaLOX* is naturally involved in defence to Phytophthora infection and was induced by chitosan treatment in cultivar 15080. However, the moderate expression of *CaLOX* when Phytophthora infection was coupled with chitosan treatment might indicate the direct action of chitosan in reducing the pathogenicity of Phytophthora.

Similar patterns of gene expression were observed for *CaPTI1* and *CaRGA2*, where Phytophthora infection significantly increased their expression, but which was repressed when coupled with chitosan application. The results suggest natural roles for *CaPTI1* and *CaRGA2* genes in defence to Phytophthora infection, and for chitosan in

decreasing the pathogenicity of Phytophthora. The *CaPTI1* gene encodes a transcription factor that belongs to ethylene responsive-factors family and is involved in defence mechanisms to *Phytophthora capsici*. The *CaPTI1* gene regulates the expression of several defence-related genes, such as *CaPR1* (SA dependent), *CaDEF1* (JA dependent) and *CaSAR82* (systemic acquired resistance), during Phytophthora infection (Jin et al., 2016). Meanwhile, repressed expression of *CaRGA2* has previously been shown to significantly reduce resistance to Phytophthora infection (Zhang et al., 2013). However, a steep increase of *CaRGA2* expression in the highly resistant cultivar, CM334, after Phytophthora infection (Zhang et al., 2013) was not similarly recorded in our study.

We also quantified the relative expression of *CaCAM1*, which is responsive to calcium during exposure to biotic stresses and is linked with reactive oxygen species (ROS) pathway and nitric oxide (Choi et al., 2009). Chitosan application significantly increased *CaCAM1* expression in the resistant cultivar in our study, but Phytophthora infection did not prompt any expression modulation in either the susceptible or highly resistant cultivar. These results indicate that only *CaCAM1* in the highly resistant cultivar, CM334, was responsive to chitosan application and that *CaCAM1* was not involved in natural defence to Phytophthora infection. Interestingly, a steep reduction of *CaCAM1* expression in the highly resistant cultivar was observed when treated with chitosan after Phytophthora infection. It is speculative to suggest that this might be a consequence of competition of chitosan action towards host gene modulation and inhibition of pathogen components, or that chitosan acts directly to inhibit pathogen infection.

CaPIK1 was also selected for gene expression analysis due to its role in chilli pepper defence towards Xcv infection. It is proposed that high expression of *CaPIK1* associates with bursts of reactive oxygen species (ROS), and further modulates the signalling required for the salicylic acid-dependent defence response to pathogen infection (Kim and Hwang, 2011). However, neither Phytophthora infection nor chitosan application was able to induce significant responses of CaPIK1 gene expression in susceptible and highly resistant cultivars.

In conclusion, chitosan was able to promote growth and enhance the resistance of chilli pepper plants to Phytophthora infection. The chitosan-treated plants grew taller, possessed more leaves and higher concentrations of chlorophyll. Foliar application of chitosan to Phytophthora-infected plants had significantly reduced disease incidence and severity indices, except in highly (CM334) and moderately resistant (LABA) cultivars. Moreover, results of quantitative expression analysis indicated that *CaLOX*, *CaPTI1* and *CaRGA2* genes were involved in defence mechanism to Phytophthora infection and that chitosan was suggested to play a direct role in reducing the pathogenicity of Phytophthora. Application of chitosan as an alternative bio-fungicide in Indonesia and other maritime countries is promising, considering the abundance of raw material and low production cost of chitosan bio-fungicide. However, further testing on fields is a prerequisite to verify how chitosan acts upon Phytophthora and to determine the practicality of application.

Materials and methods

Plant, chitosan and inoculum preparation and treatment

Highly (CM334), moderately (LABA), weakly (LADO) resistant and susceptible (15080) cultivars of chilli pepper were provided by PT. East West Seed Indonesia. The chilli seeds were planted on growth medium consisting of soil, husk, and cocopeat in a ratio of 1:1:1. Watering was given every second day, and fertiliser (GrowMore ™) was applied when the plants were 14 – 33 days old or up until they had 6 – 8 leaves. The plants were maintained in a screen-house with 60 – 70% moisture and 12hr photoperiod at 27 - 35^{o} C.

Chitosan stock solution (1% (w/v)) was prepared by dissolving chitosan powder (food grade, 85-89% deacetylation degree, PT. Biotech Surindo, Cirebon, Indonesia) in 0.7% (v/v) acetate acid overnight, with continuous stirring at medium speed and gradual pH adjustment (1M NaOH) to pH 6.4. The stock was then diluted into concentrations of 200, 600 and 1000 µg mL^{-1} by adding distilled water. The diluted chitosan solutions were sprayed weekly to the leaves in a foliar feeding technique, starting from day 14 after planting up to day 33. The chitosan particle size was determined by using Delsa™ Nano C particle Analyzer (Beckman Coulter), which recorded with an average diameter of 226 nm (submicron size).

The zoospores of Phytophthora capsici were obtained from PT. East West Indonesia in a concentration of 1.104 mL^{-1}, validated by counting in a hemocytometer (n = 3). A foliar feeding technique was used to inoculate five mL zoospores containing 5.10^{4} cells onto the plants after 33 days of growth. For genes expression analysis, the leaves were sampled before and after 72 hours of inoculation.

Physiological analysis

During chitosan treatment, physiological characteristics of plant growth, including plant height and leaf number, plus chlorophyll content were measured. Plant resistance towards Phytophthora capsici was observed on day ten post-infection by assessing the disease incidence index (DII) and disease severity index (DSI) as described by Asare-Bediako et al. (2015). DII is an indexing method that gives a score of 1 (infected plant) to 0 (uninfected) based on visual observation, whereas DSI utilises a broader range of scores from 0 – 5 based on the severity of the infection. Specifically, 0 describes no detection of visual symptoms of the infection; one indicates that infection symptoms are detected in <30% of the plant, while 2, 3, 4 and 5 shown infections in 30-50%, 50-70%, 70-90% and >90% of the plant, respectively.

Plant height was measured using a ruler, determining the length of the main stem from the base up to the tip (cm), while leaf number was hand-counted. The soil-plant analysis development (SPAD) meter (Chlorophyll meter SPAD-502 Plus, Konica Minolta) was used to assess chlorophyll content. The device quantified the relative amount of chlorophyll by measuring the transmittance of 600 - 700 and 400 - 500 nm wavebands, giving a reading in arbitrary units that is proportional to the amount of leaf chlorophyll (Loh et al., 2002). The readings were then calibrated into leaf

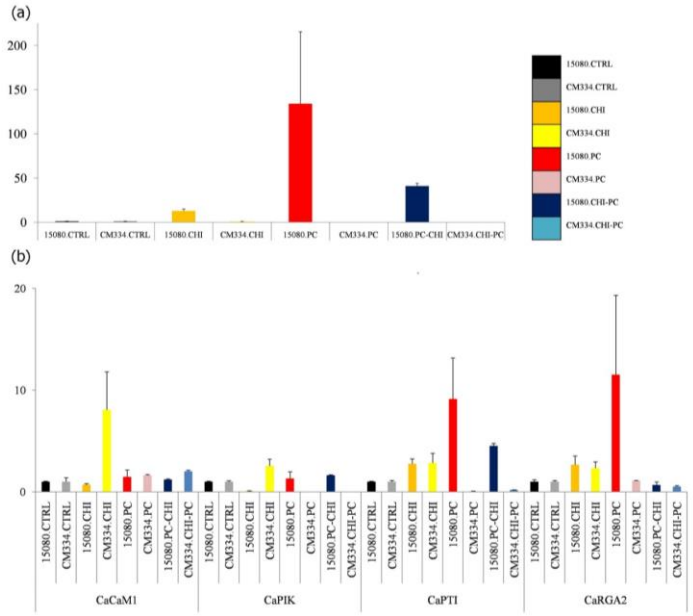

Fig 2. Quantitative analysis of defense-related gene expressions. The investigation was made to susceptible (15080) and highly resistant (CM334) cultivars with no treatment as control (CTRL), Phytophthora infected with chitosan treatment at 1000 µg mL-1 (PC.CHI) and without (PC), also chitosan treated but uninfected (CHI). A set of 5 genes were quantified, which are 9-lipoxygenase (CaLOX) (a); Ca^{2+}-bound calmodulin 1 (CaCaM1), receptor-like cytoplasmic protein kinase (CaPIK), Pto-interacting1 (CaPTI1) and resistance gene analogue 2 (CaRGA2) (b).

chlorophyll content in nmol.mg^{-1} of fresh weight, as described in Ling et al. (2011). This calibration was applied due to the high similarity of morphology between Arabidopsis and chilli pepper leaves concerning leaf thickness, waxiness, etc.

All statistical analysis of the physiological data was performed using SPSS (IBM SPSS Statistics 22.0). Analysis of variance (ANOVA) method was used to determine significance, and mean differences were adjusted with the Duncan's Multiple Range Test (DMRT) with a **P**-value <0.05.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis was performed on susceptible (15080) and highly resistant (CM334) cultivars, which were sprayed with water (i.e. control; CTRL), exposed to Phytophthora (PC) and exposed to Phytophthora infected with chitosan treatment at 1000 µg mL^{-1} (PC.CHI). Leaf tissues (consisting of three biological replicates) were collected, immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. Total RNA was isolated using the Plant Spectrum RNA Kit (Sigma-Aldrich). RNA quantity was determined using a Biophotometer (Eppendorf), and RNA quality was checked using Agilent Bioanalyzer (Agilent Technologies). Only RNA with an A260/ A280 ratio ⩾1.95 was retained for subsequent analysis. cDNA was synthesised using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions. For each sample, one µg of total RNA was retro-transcribed and diluted to a ratio of 1:5. RT-qPCR reactions were made to a final volume of 10 µL and contained one µL of cDNA. The PCR cycling program was set as follow: initial denaturation at 95° C for 3min, then 40 cycles of denaturation at 95° C for 15s and extension at 59° C for the 30s. The program completed with melt curve determination from 60° C to 95 ° C with incremental steps of 0.5° C for 5s. Expression of CaLOX, CaCaM1, CaPIK, CaPTI1 and CaRGA2 genes were quantified relative to the expression level of CaTUB and CaUbi3 using the calculation method described by Litvak and Schmittgen (2001).

All primer pairs were designed using Primer Select of the Primer3 Plus web server (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Primer pairs were designed to meet the following criteria: (1) to amplify the last two exons to avoid potential problems from truncated, non-full length cDNA; (2) to span an intron to distinguish cDNA from genomic DNA amplicons; (3) to produce amplicons between 150 - 200 bp; (4) Tm of 59.0 ± 1° C. Primers' specificity in recognising target genes in the chilli genome was validated by sequencing the amplified fragments (170 – 200 bp). Primers and sequencing results are presented in Supporting Information Table 1.

Acknowledgements

This research was funded by "Hibah Riset Strategies Nasional – 2016/17", Ministry of Research, Technology and Higher Education – Indonesia. Technical assistance from Ms Annisa Amalia in performing the quantitative analysis of gene expressions is highly appreciated.

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