# The chitinase activity of oil palm (*Elaeis guineensis* Jacq.) roots against fungal endophytes and pathogenic *Ganoderma boninense*

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**Abstract**

Application of fungal endophytes can be an alternative to control basal stem rot disease in oil palm, caused by *Ganoderma boninense*. Chitinase is a type of defensive protein synthesized by plants in response to biotic factors. The purpose of this study was to analyze the chitinase activity of oil palm as a defensive mechanism to fungal endophytes and pathogenic *G. boninense*. Four species of fungal endophytes, *Trichoderma harzianum* MTP 10 (Th-MTP10), *Trichoderma longibrachiatum* KBA 31 (Tl-KBA 31), *Lasiodiplodia venezuelensis* MJP 28 (Lv-MJP 28), *Dothidiomycetes* sp. MTD 29 (Dt-MTD 29) and one species of fungal pathogen *G. boninense* and with their each cell wall suspension were introduced to oil palm plantlets in axenic condition. Chitinase activity was observed from the root of oil palm plantlets inoculated with both living cell and cell wall suspension of endophytic fungi and pathogenic *G. boninense*. Results showed that chitinase activities varied in each fungal treatment and were significantly differed from control. Fungal cell wall elicitors were able to significantly induce chitinase activity after 1 week post treatment (wpt). Statistically, only the chitinase activity from fungal endophyte Lv-MJP 28 was significantly higher from others for 8 and 12 days. Pre-treatment of oil palm plantlet with fungal cell wall suspension for 1 wpt could induce the chitinase activity higher than control when, oil palm infected with fungal pathogen of *Ganoderma boninense*.

**Keywords :** Chitinase enzyme activity; fungal endophyte; *Ganoderma boninense*; oil palm

**Abbreviations** : wpt_week post treatment; dpt_day post treatment; wpi_week post infection, PDA_ potato dexrose agar; NAG_N-asetyl glucosamine

## Introduction

Endophytes are defined as microorganisms that spend most of their lives naturally in their host tissues with no disease symptom. Some of endophytic microorganisms are even proven to promote plant growth, fitness and protection (Hardoim et al., 2015). Despite causing no harm to their host, plants often express some genes as result of their interactions (Rosenblueth and Martínez-Romero, 2006). Plants produce their defensive system continuously as a response to the presence of these endphytic microorganisms (Schulz and Boyle, 2005). Endophytes can trigger their host defensive system intrinsically, providing additional source of defense extrinsically by producing antibiotics (Here et al., 2007). In most cases, it had been scientifically proven that plants with endophytes have shared higher defense gene expression compared to those without endophytes (Gao et al., 2010).

Every plant produced some proteins as a response to the environmental stress, abiotic or biotic (Atkinson and Urwin 2012). The plant also produced a physiological response to pathogenic (Vidhyasekaran 2008) and symbiont microorganisms (Redman et al., 2001).

Fungal endophytes or pathogens are able to switch lifestyle expression of host genotype (Redman et al., 2001). Information about the effects of endophytic fungi in inducing plant defense responses in oil palm has not yet been reported.

Fungal endophytes and parasites can induce plant defense responses such as chitinase production in oil palm tissues (Naher et al., 2012b). Research on *Atractylodes lancea* showed that endophytic fungi *Gilmaniella* sp. and their elicitor increased the activity of the enzyme phenylalanine ammonia lyase, polyphenol oxidase, chitinase and β,1-3 glucanase compared to control (Wang et al., 2012). Peroxidase, chitinase, and glucanase activities on cucumber roots were also increased after being infected with mycoparasite *Trichoderma harzianum* (Yedidia et al., 2000). Chili plants inoculated with the conidia suspension of *T. harzianum* also showed activity increment of chitinase and glucanase both on the roots and leaves (Jangid et al., 2004).

Study on resistance response of oil palm and its interaction with endophytic fungi will be a useful strategic application as biological control of oil palm diseases, particularly basal stem rot disease. The purpose of this study was to observe the effect of the introduction of living cells and the cell wall suspension of endophytic fungi and pathogenic *Ganoderma boninense* to oil palm tissues *in vitro* based on the activity of pathogenesis related protein (PR-proteins) activity namely chitinase.

## Results

### Treatments of fungal inocula

Results showed that specific activities of chitinase from oil palm roots after introduction with 4 species of living cell of fungal endophytes and *Ganoderma boninense* were significantly different at 5% after 8 and 12 days post treatment (dpt) but not at 4 dpt. Specific activities of chitinase at 4 dpt ranged from 0.039 to 0.054 U/mg protein and increased even more at 8 and 12 dpt. The living cell of Lv-MJP28 induced the highest chitinase activity at 8 dpt. The inoculation of living cell of *G. boninense* increased the specific activity of chitinase gradually during the observation, while others were not significantly different with the control treatment (Table 1).

### Effect cell wall suspension of fungal endophytes and G. boninense

Fungal cell wall (endophytes and *G.boninense*) suspension significantly elicited chitinase activity of oil palm root after 1-week post treatment (wpt) and in leaves after 2 wpt (Fig. 1). Specific activity of chitinase from the root samples 1 week post treated with the endophytic fungal cell wall and *G. boninense* ranged from 0.022 U/mg protein to 0.025 U/mg protein. Elicitor derived from different fungal cell wall had significant effect to induce chitinase enzyme activities than control only in 1 wpt (Fig 1a). The chitinase activity from leaf samples had a significant difference only after 2 weeks post treatment with cell wall from several fungal species (Fig. 1b). The chitinase enzymes activities might not affected by cell wall composition of fungal species and location of enzymes observed in plant. Overall, it can be concluded that chitinase activities of oil palm showed an increase in line with the time (Fig. 1).

The applications of fungal cell wall suspension induced the chitinase activity from oil palm both in the site (root) and off-site (leaf) of infection. Oil palm plantlets with fungal cell wall induction showed a dramatic change when they were infected with *G. boninense*, compared to control with no infection, particularly on root samples, 1 week post infection (wpi). The chitinase activity from oil palm root samples was lower and not significantly different with control 2 weeks post-infection (Fig. 2). The addition of fungal cell wall suspension was able to induce plant defense against *G. boninense* infection by producing chitinase. The overall chitinase activity in induced plantlets was higher compared to control treatment.

## Discussion

Chitinases are hydrolytic enzymes released by many plant species as a response to fungal infection. Chitinases itself are included in the 17 categories of pathogenesis-related proteins (PR-Proteins) from plants (Ebrahim et al., 2011). In this study, the oil palm samples demonstrated the ability to produce chitinase enzyme constitutively, both in control with no fungal cell wall treatment and with fungal cell wall elicitor treatment. Hamid et al. (2013) reported that there were 5 to 6 classes of plant chitinases and their activities increased gradually with the age of the plant itself. Chitinases are also produced in certain tissues of plants during specific plant development stage, such as flowers in tobacco (Neale et al., 1990) and somatic embryo of carrot (De Jong et al., 1992). Chitinases had also been reported to be produced by over 41 monocots and dicots in several organs, abscission zone, flowers, protoplasts, embryos, leaves, stems, callus and even callus suspension (Punja and Zhang, 1993).

Chitinase production in oil palm was also known to be induced when introduced with living fungal cells and fungal cell wall suspension. The chitinase activities varied depending on the species of fungi introduced. Different enzyme activities might also be due to the chitin microfibril content in fungal cell wall. Plant chitinases degrade chitin found in fungal cell wall in the infection site. Chitin or their monomers can act as an elicitor to trigger the defense response in host plants. Elicitors are normally produced by pathogens, some saprophytic fungi (Vidyasekaran, 2008), and endophytes (Gao et al., 2010). Some resistant plants often release more elicitors than susceptible plants (Vidyasekaran, 2008). When the oil palm plantlets in this study were introduced to the fungal cell wall materials, the plantlets released more amount of chitinase than control treatment. Plants recognize the cell wall component and produced specific enzymes to degrade the fungal cell wall (Lawrence et al., 2000). Resistant plants are able to lyse fungal cell wall better than susceptible plants. For instance, tomato plants that are susceptible to pathogen *Alternaria solani* constitutively produced a lower amount of chitinase compared to resistant plants (Lawrence et al., 2000). Punja and Zhang (1993) concluded that there are some inducible factors for chitinase production in plants, such as viral infection, fungal pathogens, mycorrhizae, endophytic fungi, bacteria, application of ethylene, chitosan, salicylic acid, dissolved salts, heavy metals, components of the fungal cell wall, oligosaccharides, polysaccharides, pectin, UV light, ozone, insects, nematodes, and plant injuries. However, the expression of chitinase and glucanase genes in plants can inhibit their growth during the mycorrhizal development stage in crops (Lambais and Mehdy, 1993).

Specific activity of chitinase in wheat crop susceptible to *Punicca striiformis* f.sp. *tritici,* also showed a gradual decrease after 72 hours (3 days) of pathogen inoculation, and reached the same value as control after 2 weeks post-inoculation. Specific chitinase activity in resistant plants is generally levelling up to three times higher than the response from susceptible plants, two weeks after inoculation (Mohammadi, 2002). It can be suggested that the oil palm in this study was moderately resistant, as the chitinase activity in the plantlets continued to increase up to 8 days after inoculation of pathogen *G. boninense*.

Chitinase activity from oil palm roots in this research rose slightly after being introduced to endophytic fungi Dt-MTD29. The growth rate of fungi Dt-MTD29 is possibly slower than the other fungi used in this study, causing the late chitinase response in the plant samples. On the other hand, the fungi Lv-MJP28 might have the fastest growth rate, as the plants have already started to respond on the 8th day. The highest chitinase activity as an effect of Lv-MJP28 introduction may also be related to the amount of chitin composition in its cell wall, as also in Th-MTP10, Tl-KBA31, and Gb-M fungi.

The growth rate of fungi in plant tissues can be associated with the fast response of the plant to produce chitinase. Naher et al. (2012a) reported that chitinase activity was observed to be significantly improved in the roots and leaves of oil palm 2 weeks after infection with *Trichoderma* and together with the combination of *Trichoderma* and *Ganoderma boninense*, compared to control treatment. However, the plant chitinase activity was observed to be much higher when singly infected with *Ganoderma boninense*. The chitinase activity in the oil palm induced by *T. harzianum* might work as a plant defense mechanism against pathogenic infection (Naher et al., 2012a).

**Table 1.** Chitinase specific activities of oil palm root when treated with 4 fungal endohytes and *G. boninense*.

| Fungal treatment | Chitinase specific activity of oil palm root (U.mg protein$^{-1}$) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 4 days post treatment* | | | 8 days post treatment* | | | 12 days post treatment* | | |
| Control | 0.037 | ± | 0.004 | a | 0.049 | ± 0.006 | b | 0.069 | ± 0.002 | b |
| Gb-M | 0.054 | ± | 0.002 | a | 0.071 | ± 0.017 | b | 0.098 | ± 0.013 | a |
| Th-MTP 10 | 0.052 | ± | 0.008 | a | 0.078 | ± 0.003 | b | 0.057 | ± 0.001 | b |
| Tl- KBA 31 | 0.039 | ± | 0.004 | a | 0.070 | ± 0.004 | b | 0.059 | ± 0.005 | b |
| Dt-MTD 29 | 0.039 | ± | 0.001 | a | 0.047 | ± 0.005 | b | 0.052 | ± 0.002 | b |
| Lv- MJP 28 | 0.047 | ± | 0.005 | a | 0.171 | ± 0.013 | a | 0.094 | ± 0.010 | a |

*The treatment is  followed by the notation the same letter in the same column are not significantly different at the level of 5% .
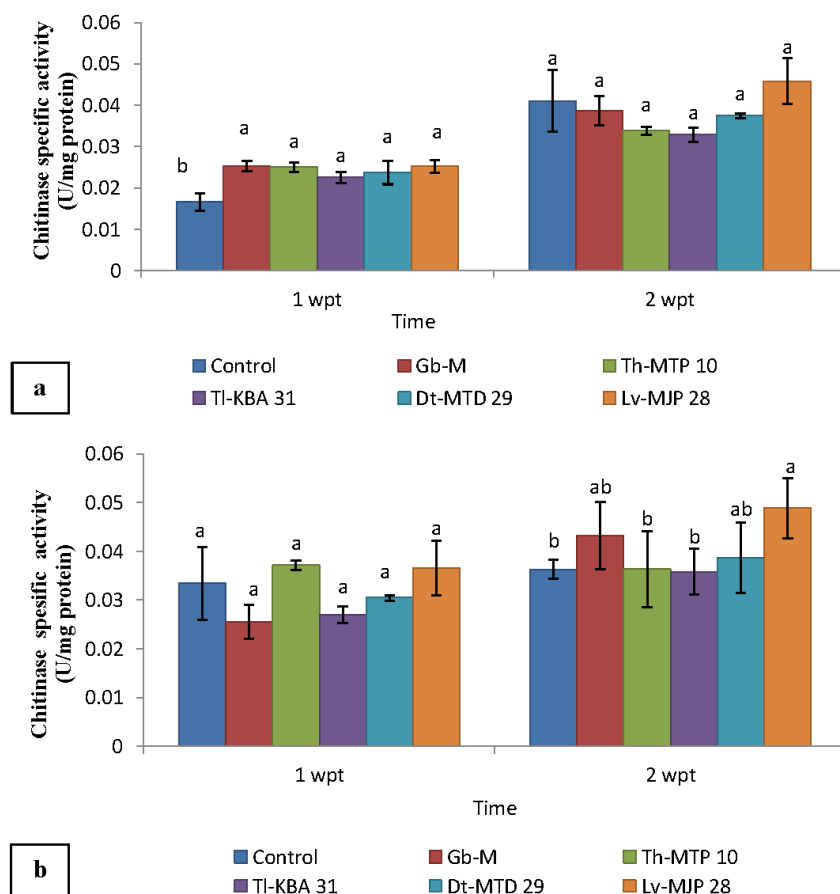




**Fig 1.** The chitinase specific activities of oil palm root (a) and leaves (b) at 1 and 2 wpt of fungal cell wall suspension treatment as an elicitor. Notation letter above the bar is related to a significantly different at the level of 5%.
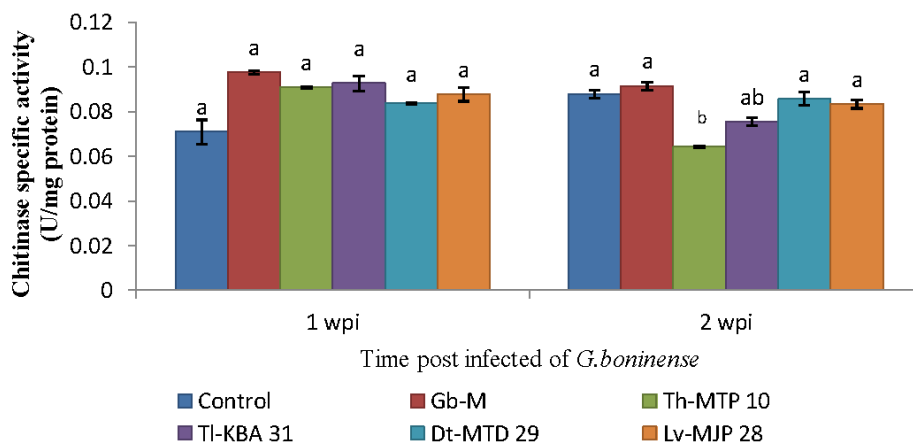


**Fig 2.** The chitinase specific activities of oil palm root against fungal cell wall, post infected with *G.boninense* at 1 and 2 wpi. Notation letter above the bar is related to a significantly different at the level of 5%.

The class 2 and 3 of chitinase gene on the oil palm leaves showed the highest expression, 2 weeks after inoculation with *T. harzianum*, higher than the inoculation of *G. boninense* and combination of *T. harzianum* and *G. boninense*. The class 1 chitinase gene showed the highest expression level, in leaves treated with *G. boninense* and *Trichoderma* 8 weeks after inoculation, showing a higher expression gene compared to the other two gene as well as the other two treatments and control (Naher et al., 2012b).

The increased chitinase level post-induced with fungal antagonists have also been reported in several studies. The roots of cucumber plants treated with *T. harzianum* showed higher chitinase, β-1,3 glucanase, cellulose, and peroxidase activity 72 hours after inoculation, compared to control (Yedidia et al., 2000). Observation on chitinase activity (PR-3) and β-1,3 glucanase in chilli plant roots also showed a gradual increase after being soaked with fungal spore suspension *T. harzianum*, with the highest activity recorded at day 10 (Jangid et al., 2004). Chitinase activities from *Atractylodes lancea* plantlets introduced to elicitor derived from endophytic fungal cell wall *Gilmaniella* sp. were slightly higher than control. The highest chitinase activities were observed to be on day 10 after treatment (Wang et al., 2012).

Chitinase showed high activities in elicited oil palm roots upon infection with pathogen *G. boninense*. Chitinase activities were detected to be higher than control one week of post-infection. However, they dropped to lower or equal to the value in control after two weeks post-infection. Chitinase in oil palm might be produced as an initial defense to *G. boninense*. Lawrence et al. (2000) stated that the enzyme activity of host plants can degrade the fungal cell wall components (as elicitor). Then the cell wall components will elicit the plant defense system. The release of cell wall components is a phenomenon in the event of pathogenesis. The amount of enzymes that liberates elicitor in the area of infection plays a role in triggering the next plant defense responses during the infection. There are several differences in the timing and amount of enzymes required to degrade fungal cell wall components between resistant and susceptible plants. According to Lawrence et al. (2000), the accumulation of chitinase and β-1,3 glucanase in susceptible tomato plants had a significant value from 8 days post-infection with *Alternaria solani*, while the resistant plants accumulated the enzymes only within two days.

The plants have several mechanisms resistance to pathogens beside to produce the pathogenesis resistance (PR) protein. Besides chitinase, plant also produced phytoalexin and lignifications process in their cell wall. The chitin as elicitor can also induce the lignifications process (Vidyasekaran, 2008).

## Materials and methods

### Fungal and plant materials

Four fungal endophytes species isolated from oil palm tissues collected from previous study by Yurnaliza et al. (2014) were used in this research. The four of fungal endophytes showed antifungal activity against *Ganoderma boninense* (Yurnaliza et al., 2014) with the species as follows: *Trichoderma harzianum* MTP 10 (Th-MTP 10), *Trichoderma longibrachiatum* KBA 31 (Tl-KBA 31), *Lasiodiplodia venezuelensis* MJP 28 (Lv-MJP 28) and *Dothidiomycetes* sp. MTD 29 (Dt-MTD 29). The pathogenic fungi *Ganoderma boninense*. Pat strain Marihat (Gb-M) was collected from Indonesian Oil Palm Research Institute (IOPRI), Medan,

Indonesia. All of the fungi culture used in this research were cultured on Potato Dextrose Agar (PDA; Merck®) medium. The axenic cultures of oil palm were obtained from Laboratory of Tissue Culture in IOPRI. The cultures were cultivated on Murashige and Skoog (MS) Agar medium in tubes and incubated on 26 °C under fluorescent light. Only plantlets free from contamination were used in this study.

### Fungal inocula treatments

The live cultures of fungal endophytes and *G.boninense* were used to induce the chitinase activity from the root samples. Two plugs of fungal mycelium from the edge of actively grown colonies on PDA medium (5-7 days) were cut with 6 mm diameters of cork-borer. They were then co-cultured together with two oil palm plantlets in a tube containing 10 ml of MS medium at pH 5.7. Control was a plantlet with no fungal treatment. The treated oil palm plantlets were incubated for 4, 8 and 12 days.

### Fungal cell wall treatment (as elicitor)

The chitinase activity was observed in oil palm plantlet cultures in interaction with fungal cell wall material. The cell wall of fungal endophytes and *G. boninense* were collected from mycelial of cultured fungi in the liquid medium of Potato Dextrose Broth (PDB; Merck®). Every fungal culture was homogenized with blender and then filtered with Whatman filter paper. Each mycelium filtrate was suspended in distilled water. The concentration of cell wall suspension was calculated by gravimetric method and then autoclaved for 20 minutes at 121 °C. The mycelial suspension in 20 % (W/V) concentration from each fungal was added to the plantlet oil palm culture in MS Broth medium. Control was the plantlets with no fungal cell wall suspension treatment (using only distilled water). The oil palm plantlet cultures were divided in 2 two group. One group was analyzed for the enzyme activities directly after treatment with fungal cell wall suspension, 1 and 2 weeks after treatments. The others were analyzed for the enzyme activity after infected with *G. boninense* for 1 week post infection (wpi).

### Protein extraction from oil palm root

Oil palm plantlet were collected at each time point and washed with running tap water. Crude protein was extracted from root according to Naher et al. (2012) method with modification. Roots from each sample were frozen in liquid nitrogen and then grinded using pestle and mortal. The homogenate samples were added to extraction buffer consisted of 100 mM of sodium acetate buffer pH 5.2 and 0.1 % mercaptoethanol in 1: 10 ratio (tissue:buffer; w/v). The crude protein was collected by centrifugation at 7,000 *g* for 20 min at 4 °C. The protein content in supernatant were measured and enzyme activity assay was assayed or stored at -20 °C.

### Assay for chitinase activity

Chitinase enzyme assay was carried out with colloidal chitin as substrate. Colloidal chitin was prepared from crabs shells (Sigma-Aldrich USA) using Skujinns et al. (1965) methods. The assay contained 0.5 ml of the crude enzyme from each sample, 0.5 ml of 0.5 % colloidal chitin in 50 mM of acetate buffer pH 5.5. The mixture was incubated at 37 °C for 2 h. The reaction was stopped by boiling the mixture for 5 min and then centrifuged at 10,000 rpm for 5 min. Sodium

tetraborate (100 µl of 0.18 M) was added to 500 µl of supernatant. The N-acetyl glucosamine (NAG) liberated from the reaction was determined using Reissig methods (Yurnaliza et al., 2001). Control was the crude enzyme without the addition of colloidal chitin substrate. The purple colour from the NAG was measured by spectrophotometer at 585 nm of wavelength. The intensity of the purple colour increased linearly with the NAG concentration in the medium and calculated by regression from the standard curve from the serial concentration of NAG. One unit of chitinase activity was determined as µmol amount NAG liberated during the determination condition. The specific activity was expressed as U/mg protein. The protein content was calculated using Lowry methods (Plummer, 1978).

### Statistical analysis

The experimental research used was completely randomized design with three replications. The data obtained were statistically analyzed by ANOVA, and the data significance was tested using Duncan test.

### Conclusion

The chitinase activities of oil palm varied as a response to fungal cell wall and their elicitors depending on the fungal species and infection sites. No significant chitinase activities were found from fungal endophytes treatment in 4 dpt. Chitinase response as a defensive mechanism was detected only when the oil palm plantlets were treated with endophytic fungal cell wall or the pathogen *Ganoderma boninense*.

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