Journal of Oil Palm Research Vol. 32 (3) September 2020 p. 464-470
DOI: https://doi.org/10.21894/jopr.2020.0033

INDICATION OF OIL PALM (*Elaeis guineensis* Jacq.) RESISTANCE TO *Curvularia* LEAF SPOT DISEASE BY PR-PROTEINS PRODUCING ABILITY

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ABSTRACT

Curvularia *leaf spot disease of oil palm seedlings caused by* Curvularia oryzae *is an important disease and widespread in South-east Asia. In this study, the resistance of oil palm* C. oryzae *was indicated after treatment with* 10^6 *spores* ml^{-1} *of* C. oryzae *NK1. The PR-proteins (chitinase and β-1,3-glucanase) were detected within 24 hr. From the inoculated oil palm seedlings, lines 129 and 187 were indicated as susceptible and resistant lines, respectively based on activities of the pathogenesis-related (PR)-proteins. The chitinase and β-1,3-glucanase activities of line 187 were 14.03 ± 0.87 and 13.51 ± 1.04 U* ml^{-1}*, while the respective activities of line 129 were much lower at 3.76 ± 0.41 and 4.31 ± 0.83 U* ml^{-1} *and not significantly different from control plant. The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed accumulation of 22, 25 and 33 kDa in inoculated line 187, whereas protein bands of line 129 were not visible. The disease symptoms in the susceptible line appeared approximately on 10% of the leaf area after 72 hr of fungal inoculation while no leaf spot symptoms were visible in the resistant line. Thus, the resistance of oil palm against* Curvularia *leaf spot disease was successfully indicated based on PR-protein producing ability, prior to testing with inoculation.*

Keywords: PR-protein, chitinase, β-1,3-glucanase, resistance, indication.

Date received: 22 February 2019**; Sent for revision:** 4 April 2019**; Accepted:** 11 October 2019**; Available online:** 30 June 2020.

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is a monoecious and cross-pollinated plant. *Tenera* hybrid variety is the result of hybridising *dura* and *pisifera* and has a high commercial value (Verheye, 2010). Indonesia, Malaysia and Thailand are the most important producers of palm oil for international trade supplying food industry, cosmetics and renewable energy. However, the main factors that should be considered for supporting oil palm production include plantation area, diseases, agronomic methods, environment and high-performance variety. Recently, *Curvularia* leaf spot disease caused by *Curvularia oryzae* is an important disease in seedlings of *tenera* hybrid variety of oil palm, in the nurseries in southern Thailand (Sunpapao *et al.,* 2014). This disease usually infects 2-3 months old oil palm seedlings and decreases both quality and quantity of the seedlings (Kittimorakul *et al.,* 2013). Chemical, biological and mechanical controls are applied to the leaf spot disease in the nurseries, but the pathogen usually attacks every year in the beginning of the rainy season.

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Higher plants possess a broad range of mechanisms to protect themselves against various threats and stresses, especially against attacks by pathogens including fungi, bacteria and viruses (Agrios, 1997). These stresses induce reactions such as producing phenolic compounds, phytoalexins and pathogenesis-related (PR) proteins that subsequently prevent invasion by various pathogens (Bowles, 1990). Among the PR-proteins, chitinase and β-1,3-glucanase are two important hydrolytic enzymes that are produced in many plant species after infection by various pathogens (Ebrahim *et al.*, 2011). The chitinases and glucanases are thought to limit fungal growth as chitin and glucans are widely distributed in the cell walls of fungal pathogens. This anti-fungal function has been demonstrated *in vitro* against several fungal pathogens not only on plant leaf diseases but also on root diseases such as black spot disease caused by *Alternaria brassicicola* in rocket salad (Gupta *et al.*, 2013), leaf blight pathogen (*A. palandui*) in onion (Karthikeyan *et al.*, 2005), late blight disease caused by *Phytophthora infestans* in tomato (Anfoka and Buchenauer, 1997), necrogenic fungus (*Colletotrichum lagenarium*) of cucumber leaves (Ji and Kie, 1996) and root rot disease of cocoyam caused by *Pythium myriotylum* (Nyochembeng and Beyl, 2015). Thus, these enzymes are important determinants of the resistance of plants to fungal diseases (Funnell *et al.*, 2004). Chitinase and β-1,3-glucanase have synergistically stronger anti-fungal activity against a wider range of fungi when used in combination than when acting singly (Mauch *et al.*, 1988).

In oil palm breeding programme, aside from palm oil yield, disease resistance is also an important characteristic required of a high performance variety. Oil palm basal stem rot caused by *Ganoderma* sp. has been widely reported in oil palm plantations and included in oil palm breeding programmes (Idris *et al.*, 2004; Durand-Gasselin *et al.*, 2005). However, there are only a few studies on *Curvularia* leaf spot disease, and most have focused on chemical and biological control. Therefore, the purpose of this current study was to indicate, based on PR-protein producing ability, oil palm lines resistant to leaf spot disease (*C. oryzae*).

MATERIALS AND METHODS

Plant Materials

Ten different genotypes of oil palm *tenera* hybrid (*Dura* x *Pisifera*), lines 117, 129, 138, 155, 177, 187, 188, 202/6, 203 and 207, were initially selected from 124 lines by detached leaf method. Then, seedlings of these 10 genotypes were subjected to pathogenicity test to study their resistance against *C. oryzae* at the Oil Palm Agronomical Research Centre: Phase 2, Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University, Thailand (Kittimorakul *et al.*, 2019) to separate resistance and susceptible lines before further testing for enzyme activities. The seedlings chosen for this study were 3-4 months old, raised in the greenhouse conditions with temperature varying between of 25°C-30°C, 60% ± 5% relative humidity (RH), and 12 hr photoperiod. The experiments followed a completely randomised design (CRD) with four replicates per line (one seedling per one replicate).

Fungal Culture and Inoculation

The virulent strain *C. oryzae* NK1 (Kittimorakul *et al.*, 2014) was used to inoculate oil palm seedlings. The fungus was transferred from potato dextrose agar slants to corn meal agar, and incubated at room temperature (25°C-28°C) for 20 days. Spore suspensions were prepared with sterilised distilled water and adjusted to 1 x 10^6 conidia ml^{-1} under aseptic conditions. To inoculate, the oil palm seedlings were each sprayed with 20 ml spore suspension, while plants sprayed with sterile distilled water served as uninoculated controls. Seedlings were covered with plastic bags and incubated for 48 hr to stimulate the pathogen infection.

Crude Enzyme Extraction

Forty-eight hours after inoculation, 1 g of oil palm leaf from each plant was sampled for crude enzyme extraction. The tissue was crushed in a cooled mortar with pestle, in 5 ml of 50 mM potassium phosphate buffer (pH 7.0) for chitinase and with 50 mM acetate buffer (pH 5.0) for β-1,3-glucanase. Then, the leaf extracts were transferred to microcentrifuge tubes (1.5 ml) and centrifuged at 10 000 rpm for 5 min. The supernatant (crude enzyme) was transferred to a new microcentrifuge tube and stored at 2°C-4°C until use in enzyme activity determination.

Chitinase Assay

The reaction mixture consisted of 250 µl sample and 250 µl of 1% colloidal chitin as a substrate in 50 mM potassium phosphate buffer at pH 7.0. The mixture was incubated at 37°C for 30 min. After incubation, 500 µl of dinitrosalicylic acid solution (DNS) was added. The enzyme activity was stopped by heating the mixture to 100°C for 15 min, and then cooled at room temperature. Then, 4 ml of distilled water was added and the enzyme activity was determined by measuring the absorbance at 575 nm. One unit (U) of chitinase activity was defined as releasing 1 µmol of N-acetyl-D-glucosamine from the substrate per min.

β-1,3-glucanase Assay

The β-1,3-glucanase activity was measured using laminarin as a substrate. The reaction mixture (250 µl) consisted of 125 µl sample and 125 µl of 1% laminarin in 50 mM acetic acid buffer at pH 5.0. The mixture was incubated at 37°C for 30 min. After incubation, 250 µl of DNS was added. The enzyme activity was stopped by heating the mixture to 100°C for 15 min and then cooled at room temperature. Then, 2 ml of distilled water was added. Enzyme activity was determined by measuring the absorbance at 550 nm. One unit (U) of β-1,3-glucanase activity was defined as releasing 1 µmol of glucose from the laminarin per min.

Chitinase and β-1,3-glucanase Activities from the Selected Oil Palm Seedlings

The oil palm seedling lines were selected based on the previous experiment (one line from the highest activities and one line from the lowest activities) and evaluated for activity time-profiles of two PR-proteins, chitinase and β-1,3-glucanase. One gram fresh weight leaf samples were collected from the selected lines at 0, 24, 48, 72, 96, 120, 144 and 168 hr after inoculation with the fungal pathogen. The samples were extracted and centrifuged. Crude extracts were measured for the enzyme activities.

Protein Assay

The total protein in oil palm leaf extracts was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard. The reaction mixture containing 0.1 ml of sample and 5 ml of protein reagent [Coomassie Brilliant Blue G-250 dissolved in 95% ethanol and 85% (w/v) phosphoric acid] was incubated for 5 min and the developed colour was determined at 595 nm.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Crude extracts from oil palm leaves were applied to mini-gels (12% separating gel and 4% stacking gel) to determine proteins by molecular mass in the crude extracts, according to the procedure of Laemmli (1970). Samples of approximately 20 µl were loaded in each well and electrophoresis was performed at 100 V constant voltages for the stacking gel (15 min) and at 120 V for the separating gel (45 min). The proteins were fixed and stained for 15 min in Coomassie blue (R-250) staining solution. After staining, the gels were washed with a destaining solution and slowly shaken on a horizontal rotator for about 10 min. The destaining solution was refreshed twice. Then, the samples were incubated overnight in distilled water. The molecular masses of resolved proteins were estimated by electrophoresis of the marker proteins (BioLabs Inc.) with molecular masses ranging within 11-245 kDa.

Statistical Analysis

The enzyme activities (mean values) were subjected to analysis of variance (ANOVA) using the generalised linear model (GLM) procedure and Tukey's HSD test ($P \leq 0.05$) in SPSS software (Version 16.0).

RESULTS AND DISCUSSION

Chitinase and glucanase are produced in many higher plant species after induction by fungal inducers. These enzymes can inhibit fungal growth and play a role in self-defense (Ham *et al.*,1991) because the major components of fungal cell walls are the polysaccharides, chitin and glucan, which are the substrates for chitinase and glucanase (Sela-Buurlage *et al.,* 1993). Prior studies have reported that when coconut (*Cocos nucifera* L.) root was treated with *Pseudomonas fluorescens*, *Trichoderma viride* and *T. harzianum* in combination with chitin, it produced chitinase and β-1,3-glucanase against *Ganoderma lucidum* (Karthikeyan *et al.,* 2006). Chairin and Petcharat (2017) reported that in longkong fruit (*Aglaia dookkoo* Griff.) chitinase and β-1,3-glucanase were detected in peel extracts after exposure to the fungus *Metarhizium guizhouense,* and these inhibited the mycelial growth of fruit rot fungi, *Fusarium* sp. and *Botrytis* sp. In this current study, oil palm plants were treated with *C. oryzae* and this activated PR-proteins, chitinase and β-1,3-glucanase. Forty-eight hours after the fungal inoculation, the oil palm lines 138 and 187 showed high chitinase activities at 14.88 ± 1.31 and 17.84 ± 1.46 U ml^{-1}, respectively, and β-1,3-glucanase activities at 12.02 ± 1.03 and 14.23 ± 1.31 U ml^{-1}, in the same order. lines 138 and 187 were not significantly different at $P \leq 0.05$. In contrast, lines 117, 129 and 155 presented low activities of both enzymes and were not significantly different ($P \leq 0.05$) from the uninoculated controls in β-1,3-glucanase activity (*Table 1*). Not only for enzyme activities results, but Kittimorakul *et al.* (2019) also reported that lines 117, 129 and 155 were susceptible lines because of rapid manifestation of disease symptom (three days after inoculation), high disease score (3.10, 4.55 and 3.70) and high percentage of disease incidence (62%, 91% and 74%). Based on the enzyme activity results, two candidate lines were selected from the 10 lines tested. Oil palm line 187 was selected for its high enzyme activities, while line 129 was selected for its comparatively low activities. These selected oil palm lines were then assessed for the time profiles of enzyme activity, for

proteins with SDS-PAGE, and for disease symptom occurrence.

The chitinase and β-1,3-glucanase activities of the selected oil palm lines were determined every 24 hr over seven days after inoculation with the fungus. It was found that the chitinase activity of line 187 was 14.03 ± 0.87 U ml^{-1} at 24 hr, peaked to 16.84 U ml^{-1} at 48 hr and then decreased continuously to 5.77 U ml^{-1} at 168 hr. For line 129, chitinase activity at 3.76 ± 0.41 U ml^{-1} was observed at 24 hr and it decreased slightly to 1.25 U ml^{-1} at 168 hr after inoculation (*Fig*ure *1a*). Regarding β-1,3-glucanase, the enzyme activity in oil palm line 187 at 24 hr after inoculation was 13.51 ± 1.04 U ml^{-1}, the highest at 15.02 U ml^{-1} activity was at 48 hr, and the activity then decreased continuously to 4.50 U ml^{-1} at 168 hr. In contrast, the β-1,3-glucanase activity of line 129 (4.31 ± 0.83 U ml^{-1} at 24 hr) was not significantly different from its uninoculated control (*Figure 1b*). The proteins in oil palm leaves were separated by SDS-PAGE and stained with Coomassie blue R-250 to observe the protein bands. The SDS-PAGE analysis showed differences in the expression of major proteins in the selected oil palm lines 129 and 187 from their uninoculated controls. Oil palm line 129, which produced low activities of chitinase and β-1,3-glucanase, did not have visible protein bands. However, line 187 had protein bands for 22,

TABLE 1. ENZYME ACTIVITIES (mean ± standard deviation) OF OIL PALM LEAF EXTRACTS 48 hr AFTER INOCULATION

Line number	Chitinase (U ml^{-1})*		β-1,3-glucanase (U ml^{-1})*	
	Control plants	Fungal treated plants**	Control plants	Fungal treated plants**
117	2.26 ± 0.19^{B}	6.85 ± 1.40^{Adef}	2.04 ± 0.18^{A}	3.50 ± 1.07^{Afg}
129	0.41 ± 0.16^{B}	3.27 ± 0.27^{Af}	0.90 ± 0.19^{A}	2.05 ± 0.70^{Ag}
138	3.11 ± 0.74^{B}	14.88 ± 3.62^{Aab}	2.98 ± 0.09^{B}	12.02 ± 1.03^{Aab}
155	1.48 ± 0.36^{B}	4.61 ± 0.60^{Aef}	1.97 ± 0.21^{B}	4.91 ± 0.16^{Aef}
177	2.89 ± 0.79^{B}	7.16 ± 1.15^{Ade}	1.79 ± 0.33^{B}	7.04 ± 1.00^{Ade}
187	3.36 ± 0.95^{B}	17.84 ± 1.46^{Aa}	4.00 ± 0.09^{B}	14.23 ± 1.31^{Aa}
188	3.34 ± 0.80^{B}	12.56 ± 1.30^{Abc}	3.17 ± 0.61^{B}	10.09 ± 1.47^{Abc}
202.6	2.25 ± 0.50^{B}	9.76 ± 0.78^{Acd}	1.89 ± 0.11^{B}	8.18 ± 0.79^{Acd}
203	3.05 ± 0.67^{B}	13.57 ± 1.07^{Ab}	2.36 ± 0.39^{B}	11.70 ± 1.36^{Ab}
207	3.17 ± 0.15^{B}	12.51 ± 1.03^{Abc}	2.88 ± 0.97^{B}	11.17 ± 0.83^{Ab}

Note: * Same capital superscript in a row indicates no significant difference by Least Significant Difference test ($P \leq 0.05$).
** Same lower case superscript in a column indicates no significant difference by Least Significant Difference test ($P \leq 0.05$).

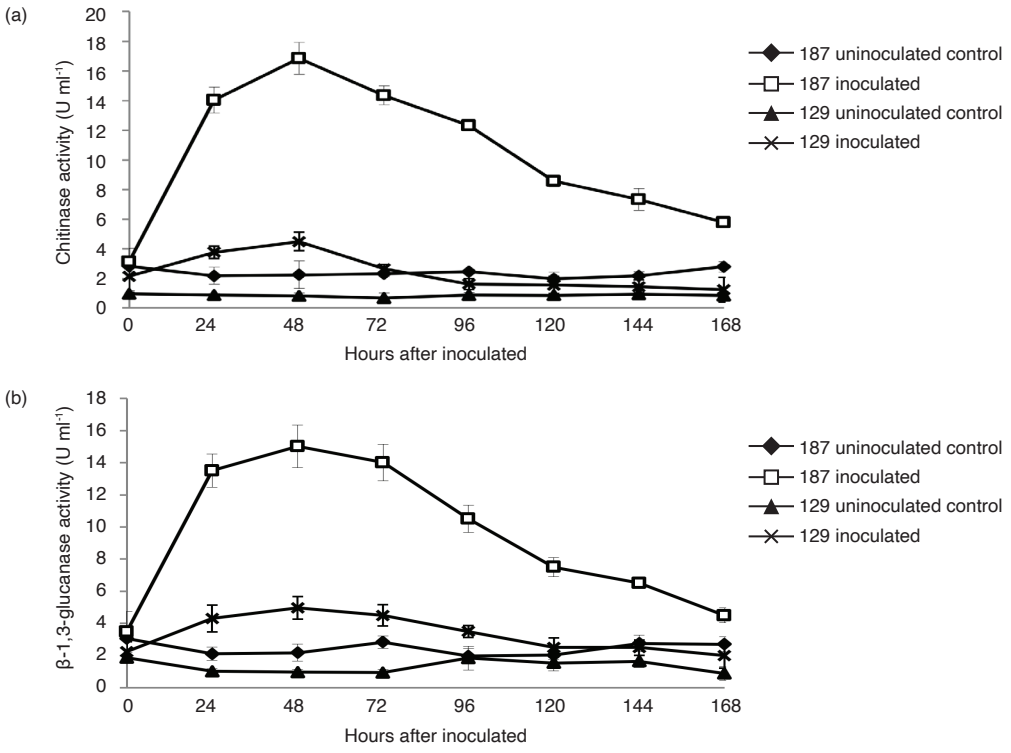

Figure 1. Enzyme activities of oil palm leaf extracts in control and Curvularia *inoculated cases: (a) chitinase activity, and (b) β-1,3-glucanase activity.*

25 and 33 kDa at 24 hr, and the bands were darker at 48 hr after fungal inoculation (*Figure 2*). Hegde and Keshgond (2013) reported that most plant chitinases have molecular mass in the range from 15 to 43 kDa, and plant glucanases have molecular mass from 33 to 44 kDa. In addition, Syahanim *et al.* (2013) reported that several proteins with molecular weights of less than 50 kDa were expressed in oil palm root on Days 3 and 7 after *Ganoderma boninense* infection, and one of these was glucanase.

The observations of PR-proteins, chitinase and β-1,3-glucanase in this study were related to disease symptom occurrence that also differed between oil palm lines 129 and 187 after *Curvularia* inoculation. In the high enzyme activity line 187, no disease symptoms were visible at 72 hr after fungal inoculation. In contrast, line 129 with low activities of PR-proteins had small light brown lesions, 1-2 mm in size, covering approximately 10% of leaf area (*Figure 3*). In the disease symptom index of Kittimorakul *et al.* (2014), the rapid appearance of small lesions over approximately 1%-10% of leaf area distinguishes the susceptible lines, while the genotypes that have no or delayed lesions on leaves are considered resistant. Based on our observations on PR-proteins and disease symptoms, oil palm line 129 was susceptible while line 187 was resistant in that indexing system.

Analogous observations by Pareek *et al.* (2013) show higher chitinase and β-1,3-glucanase activities response to the fungal pathogen *Macrophomina phaseolina* in the resistant moth bean cultivar (FMM-96) over the susceptible cultivar (RM0-40 and CZM-3). Anguelova-Merhar *et al.* (2001) found that the wheat plant resistant line (Kareee/Lr35) had higher chitinase and β-1,3-glucanase activities after inoculation with leaf rust pathogen (*Puccinia recondite* f. sp. *tritici*) than the susceptible line (Karee). In this current study, PR-protein activation could be detected within 24 hr after *Curvularia* inoculation, before the disease symptoms that appeared at 72 hr for the susceptible line, and not before 168 hr for resistant line. The early enzyme activities significantly differed between the resistant and susceptible lines. Thus, PR-protein producing ability could be used to indicate oil palm lines resistant to *Curvularia* leaf spot disease, independently of disease symptom occurrence.

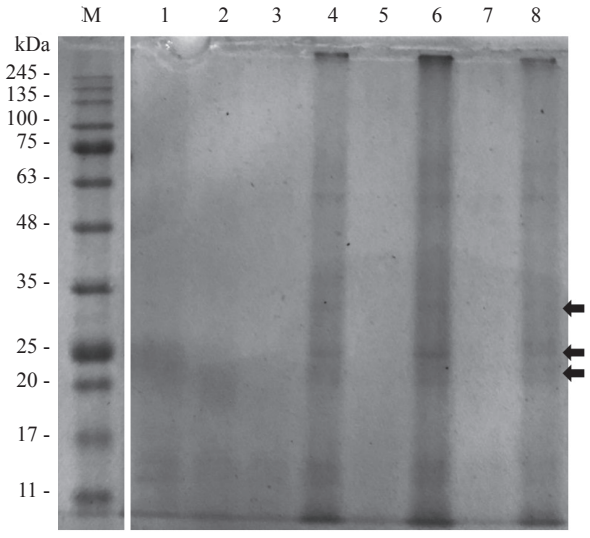

Figure 2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) protein bands for oil palm leaf extracts after Curvularia *leaf spot inoculation. Lane M: protein marker, lane 1: uninoculated control of line 129, lane 2: uninoculated control of line 187, lanes 3, 5 and 7: line 129 at 24, 48 and 72 hr, respectively, and lanes 4, 6 and 8: line 187 at 24, 48 and 72 hr, respectively.*

Figure 3. Curvularia *leaf spot disease symptoms at 72 hr after fungal inoculation; line number 187 (left) and line number 129 (right).*

CONCLUSION

PR-proteins, chitinase and β-1,3-glucanase, in oil palm seedlings were induced by exposure to the fungal pathogen *C. oryzae,* and their activation levels could be used to indicate oil palm lines resistant to *Curvularia* leaf spot disease within 24 hr after exposure, well before the appearance of any disease symptoms. Three lines (138, 187, and 203) showed high enzyme activities, while line 129 was the susceptible line that presented the lowest enzyme activities. When the proteins in oil palm leaves of lines 187 and 129 were separated by SDS-PAGE, line 187 expressed the protein bands with molecular weight ranges of chitinase and β-1,3-glucanase after fungal inoculation, but line 129 did not have visible protein bands. This study suggested that three oil palm *tenera* hybrid genotypes (138, 187 and 203) were the candidates to test field performance for disease resistance under farmers' commercial nursery conditions and also candidates for breeding and developing new oil palm varieties resistant to *Curvularia* leaf spot disease.

ACKNOWLEDGEMENT

This work was supported by the government budget of Prince of Songkla University, PSU Ph.D graduate scholarship, Oil Palm Agronomical Research Centre: Phase 2 and Centre of Excellence in Agricultural and Natural Resources Biotechnology, Prince of Songkla University. Moreover, we are grateful to Associate Professor Dr Seppo Karrila, Faculty of Science and Industrial Technology, Prince of Songkla University, Surat Thani Campus, for helping to improve the manuscript.

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