AENSI Journals

Advances in Environmental Biology

ISSN-1995-0756 EISSN-1998-1066

Journal home page: http://www.aensiweb.com/AEB/

Fatty acids and Phenols Involved in Resistance of Oil Palm to *Ganoderma boninense*

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ARTICLE INFO

Article history:
Received 12 February 2015
Accepted 1 March 2015
Available online 28 March 2015

Keywords:
Basal stem rot, oil palm, phenols, fatty acids

ABSTRACT

A study has been conducted to analyse volatile organic compounds, which may be involved in the resistance of oil palm to *Ganoderma boninense.* Four sixteen month-old commercial oil palm progenies Deli dura x AVROS pisifera crosses (P1, P3, P4 and P5) were inoculated using rubber wood blocks colonized with *G. boninense.* The palms were harvested nine months post-inoculation and were evaluated for Disease Incidence and Disease Severity. Differences of volatile organic compound profiles between the non-inoculated and inoculated seedlings were studied using gas chromatography mass spectrometry (GC-MS). The GC-MS analysis revealed the presence of eight major phytocompounds with higher levels of several fatty acids such as benzoic acid, methyl ester; 1, 4-benzenedicarboxylic acid, dimethyl ester; hexadecanoic acid, methyl ester; 9,12-octadecadienoic acid (Z,Z)-, methyl ester; 9-octadecenoic acid (Z)-, methyl ester and octadecanoic acid, methyl ester. The other two phenol compounds present were phenol, 2,6-dimethoxy- and phenol, 2,4-bis (1,1-dimethylethyl). The identified compounds were examined for their feasible roles in plant defense against pathogen-related stress and their prospective application as biomarkers for evaluating oil palm progenies for the future development of resistant varieties against Basal Stem Rot.

To Cite This Article: Rozlianah FS, Jualang AG and Chong KP. Fatty Acids and Phenols Involved in Resistance of Oil Palm to *Ganoderma boninense*. ***Adv. Environ. Biol., 9(7),*** *11-16, 2015*

INTRODUCTION

Oil palm cultivation in Asia has grown from approximately 200, 000 ha in the 1960's to 6.5 million ha in 2005, making up 90% of world palm oil production [6], Malaysia being the second world's largest producers of palm oil. A fungal disease called Basal Stem Rot (BSR) caused by *Ganoderma boninense* results in significant yield reduction in the annual harvest [11,5,4]. The most severe losses from BSR occur in Indonesia and Malaysia whereas lower incidences are recorded in Africa, Papua New Guinea and Thailand [12]. The disease is usually associated with mature stands; however, incidences were also recorded on palms as young as 12-24 months after planting [20]. The disease can cause the death of up to 80 % of the plantation by the time the palms are halfway through their economic life span [21,22,18]. Roslan and Idris [18] reported that *Ganoderma* attack can lead to yield reduction of fresh fruit bunch (FFB) at the rate of 0.04 tonnes to 4.34 tonnes/ ha at 10 to 22 years of planting respectively. Based on the *Ganoderma* incidence annual growth rate, it is estimated that in 2020, a total of 400 thousand hectares (65.6 million palm trees) could be affected by yield reduction. Numerous trials have been reported in an attempt to detect *Ganoderma* infection at the early stages and to control the disease but to date, no reliable method is available. A promising approach may be to search for potential biomarkers for future breeding programs.

To search for reliable biomarkers, a further understanding of host-pathogen interaction is needed. Therefore, it may be beneficial to study the metabolome comprehensively [7]. Plant disease occurs when there is a compatible plant-pathogen interaction and as a result, plants release secondary metabolites as defensive compounds. Secondary metabolite profiling emphasizes the important roles of certain metabolites in plant defense mechanisms against pests, fungi and bacteria [10,15]. Several studies were reported on metabolomics that could help advance our understanding of plant metabolites that may contribute to plant susceptibility and resistance to pathogens [9,1,13]. Therefore, the objective of this study was to identify specific volatile compounds produced by oil palm seedlings that might potentially be applied as biomarkers to distinguish levels of resistance in oil palm seedlings against *G. boninense*.

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MATERIALS AND METHODS

Fungal isolates:

G. boninense culture was obtained from Genetic Lab, Sustainable Palm Oil Research Unit (SPOR) of Universiti Malaysia Sabah. It was previously isolated from Langkon Estate, Sabah, Malaysia and identified by Chong *et al*. (2011).

Plant materials:

Four sixteen month-old oil palm progenies (P1, P3, P4 and P5) of commercial Deli dura × AVROS pisifera crosses were used in this study and maintained in a nursery (shaded with one layer of black net 70%) at the Universiti Teknologi MARA, Sabah.

Preparation of rubber wood blocks and inoculation of oil palm seedlings:

Rubber wood blocks (RWB) were cut to 12 cm x 6 cm x 6 cm and treated as described by Sapak *et al*. (2008) with slight modifications. Colonized RWB were then physically attached to the roots of sixteenth month-old palm seedlings to assist with the infection. Treatments consisted of non-inoculated seedlings serving as controls, and inoculated seedlings using *G. boninense*-colonized RWB. Seedlings were arranged in randomized complete block design (RCBD) consisting of ten replicates in each treatment. The seedlings were maintained with regular watering and monthly manuring with NPK Blue 12:12:17:2 + TE for 36 weeks.

Analysis of BSR:

Disease development was observed and recorded as Disease Incidence (DI) percentage at monthly intervals for a period of nine months. DI refers to the number of seedlings visually assessed as diseased (stunted growth, foliar symptoms of chlorosis and necrosis of leaves, with or without production of basidiocarp and unopened spears) as described by Campbell and Madden (1990). The formula is as follows:

$$DI: \frac{\text{number of seedlings infected}}{\text{total number of seedlings assessed}} \times 100$$

Infection was further verified with isolation of *G. boninense* on *Ganoderma* Selective Medium from fresh infected root (tissue segment 1 cm) after surface sterilization as described by Rozlianah and Sariah (2006). To confirm that the symptoms on the seedlings were caused by *G. boninense*, the bole of the affected seedlings were dissected longitudinally for internal symptoms assessment at nine months after inoculation. The disease severity of the internal symptoms on bole tissues was assessed based on the following formula and severity scale as described by Sapak *et al*. (2008);

$$DS_{(internal)} = \frac{\text{number of seedlings in the rating x rating number}}{\text{total number of seedlings assessed x highest rating}} \times 100$$

Severity scale;
0 = healthy: no internal rot
1 = 20% rotting of tissues
2 = 20 to 50% rotting of tissues
3 = > 50% rotting of tissues
4 = > 90% rotting of tissues

Extraction of volatile compounds:

Fresh root samples (100 g) of oil palm seedlings were collected after each treatment. Using liquid nitrogen, the samples were homogenized by grinding with a mortar and pestle to a fine powder prior to metabolite extraction. The homogenized root samples (8 g) were mixed with a sufficient amount of anhydrous sodium sulphate (Merck) before being placed in a Falcon tube containing 40 mL of methanol and left for two days at 4 °C. The samples were filtered through a 0.45 µm nylon syringe filters and evaporated to dryness under reduced pressure using a rotary evaporator at 38^{o}C (Stuart RE300DB). The dried samples were diluted with HPLC grade methanol to make a final concentration of 1 mg/ mL. The samples were filtered through 0.45 µm nylon syringe filters and kept at 4 ^{o}C prior to GC-MS analysis.

Gas chromatography-mass spectrometry (GC-MS) analysis:

An Agilent Technologies 6890N Network GC system equipped with Agilent Technologies 5973N Network Mass Selective Detector was used to carry out GC-MS analysis. Chromatography was carried out with a 30 m x 0.25 mm internal diameter, HP5-MS column with 0.25 µm film thickness. The carrier gas, helium, was used at a constant flow rate of 1.25 mL min^{-1}. Injection temperature was maintained at 280^{o}C; 300^{o}C for interface temperature and 230^{o}C ion source temperature. The temperature program was set at 70^{o}C for 0.5 minutes, raised to 150^{o}C at a rate of 30^{o}C min^{-1} and finally from 150^{o}C min^{-1} to 300^{o}C at a rate of 5^{o}C min^{-1} and held for 5 min.

About 2 µL of the diluted sample was injected using the splitless mode with a mass scan range from 45-600 m/z.

Metabolite fingerprint and compound identification:

The observed spectra of each peak for the oil palm progenies were compared with three of the top choices in NIST08 Mass Spec Library to confirm the identity of the compounds based on the retention time (RT) and the relative amount (RA) of individual component expressed as percentages of the peak area relative to the total peak area.

Statistical analysis:

The non-parametric test (Kruskal-Wallis *H*-test) was used because the percentage values for DI were not normally distributed. Based on the significance from the Kruskal-Wallis *H*-test, comparison between treatments was analyzed with Mann-Whitney *U*-tests at $p \leq 0.05$. Meanwhile, data values for DS (internal symptoms of bole tissues) in different oil palm infected progenies were analyzed using a one-way analysis of variance (ANOVA) to determine statistical differences and the means were compared using the Tukey's Studentized Range (HSD) comparison method at $p \leq 0.05$ (SPSS Version 21).

Results:

BSR incidence on the inoculated oil palm seedlings:

The DI was gradually increased and started to show significant infection among the tested progenies at 6 months after inoculation (

Table 1). At the end of the experiment (9 months after inoculation) DI were significantly higher with 100% of P1 and P3 seedlings infected by *G. boninense* as compared to P4 with only 60% infected seedlings. However, the P5 infected seedling (70%) was not significantly different from P1, P3 and P4. These results revealed that P4 is a tolerant progeny in comparison with P1 and P3, but not significantly different with P5. DI was not recorded in all non-inoculated oil palm progenies seedlings.

Table 1: Disease incidence (DI) of different oil palm progenies based on foliar symptoms at monthly interval for the period of nine months after inoculation.

Oil Palm Progeny	Disease incidence (after months) (%)								
	1	2	3	4	5	6	7	8	9
P1 inoculated	0^a	0^a	0^a	10^a	40^a	70^a	90^a	100^a	100^a
P3 inoculated	0^a	0^a	0^a	20^a	50^a	80^a	90^a	100^a	100^a
P4 inoculated	0^a	0^a	0^a	0^a	10^a	20^b	40^b	50^b	60^b
P5 inoculated	0^a	0^a	0^a	0^a	10^a	30^{ab}	50^{ab}	70^{ab}	70^{ab}

Means within each column followed by the same letter are not significantly different at $p \leq 0.05$ after Kruskal-Wallis *H*-test followed by Mann-Whitney *U*-test. The values are the means of 10 replicates.

DS values for the internal symptoms of bole tissues were significantly different and much higher for P1 and P3 in comparison with P4 and P5 (Table 2). These results suggest that P1 and P3 are very susceptible progenies as the rate of the spread of *Ganoderma* revealed an extensive rotting of the bole extending into the stem. All inoculated progenies exhibited internal disease symptoms and *Ganoderma* was successfully re-isolated from the infected roots when plated on *Ganoderma* Selective Medium (GSM). Symptoms of the infected seedlings are shown in Figure 1.

Table 2: Disease severity (DS) of different oil palm progenies based on internal symptoms nine months after inoculation.

Oil Palm Progeny	Disease severity of internal symptoms of bole tissues
P1 inoculated	7.50^a
P3 inoculated	7.75^a
P4 inoculated	4.75^b
P5 inoculated	5.00^b

Means within each column followed by the same letter are not significantly different at $p \leq 0.05$. The values are the means of 10 replicates.

Detection of volatile compounds from oil palm roots:

GC-MS analysis of the crude extracts of oil palm roots revealed the presence of eight phytocompounds with higher levels of several fatty acids and only two compounds from the phenol group. Generally, lower levels of compounds were present in the non-inoculated oil palm progenies as compared to the inoculated oil palm progenies (Table 3). In general, secondary metabolites produced by infected oil palm were relatively higher for tolerant progenies, P4 and P5, namely benzoic acid, methyl ester; hexadecanoic acid, methyl ester; 9,12-octadecadienoic acid (Z,Z)-, methyl ester and 9-octadecenoic acid (Z)-, methyl ester. In this study, metabolites common in both susceptible and tolerant progenies but with higher abundance in tolerant progenies was the main criteria used to discriminate resistance level in oil palm against *G. boninense*. The GC-MS profile of P4 non-inoculated and *Ganoderma* inoculated oil palm seedlings is shown in Figure 2.

Discussion:

The pathogenicity of *G. boninense* against sixteen month-old of four different oil palm progenies (with different genetic background) was successfully conducted. The finding from the current study suggested P4 and P5 are tolerant progenies against BSR as compared to P1 and P3, which are very susceptible. To date, resistant varieties of palm against *G. boninense* is still not available. All inoculated progenies tested in this study had different levels of infection, as measured by Disease Incidence and Disease Severity with internal symptoms of bole tissues. The visual foliar symptoms for BSR were observed, including stunted growth, chlorosis and necrosis of leaves. Seedlings infected with *G. boninense* were further confirmed when several affected palms developed basidiocarp at seven months after inoculation and revealed necrosis on the longitudinal sections of the dissected bole at the end of the experiment. Both susceptible and tolerant progenies produced volatile compounds, with a higher abundance in tolerant progenies. Upon pathogenic attack, plants usually divert their metabolic pathways from primary metabolite production to producing more defense-related compounds.

Fig. 1: Healthy bole tissues of non-inoculated oil palm seedlings (A). Internal symptom of longitudinal section of oil palm bole tissues assessed nine months after inoculation (B). Development of *G. boninense* fruiting body at seven months after inoculation (C).

Table 3: List of compounds detected with GC-MS in non-inoculated and inoculated oil palm progenies.

No.	Compound name	Chemical Group	Retention time (min)	[a]Relative amounts (%)				[b]Relative amounts (%)			
				P1	P3	P4	P5	P1	P3	P4	P5
1.	Benzoic acid, methyl	FA	3.54 – .55	ND	ND	0.98	2.11	0.55	1.00	2.40	1.46
2.	Phenol, 2,6-dimethoxy	PH	5.33 – .34	1.13	ND	0.93	0.73	1.06	2.01	1.06	1.69
3.	1,4-	FA	6.93 – .94	6.89	2.21	7.61	5.98	14.12	9.52	11.16	10.6
4.	Phenol, 2,4-bis(1,1-	PH	6.99	2.00	ND	1.10	1.16	ND	ND	ND	ND
5.	Hexadecanoic acid,	FA	13.41	12.03	5.68	8.40	8.06	9.22	8.00	9.81	11.8
6.	9,12-Octadecadienoic	FA	16.35	5.78	4.28	5.41	5.65	ND	2.63	6.76	7.32
7.	9-Octadecenoic acid	FA	16.45	3.90	2.81	4.66	5.39	ND	ND	6.87	7.73
8.	Octadecanoic acid,	FA	16.89	10.04	4.97	5.08	4.15	13.67	11.01	5.36	4.66

Notes: PH = Phenol
FA = Fatty acid
ND = Not detected
[a]non-inoculated oil palm progenies
[b]inoculated oil palm progenies

Certain fatty acids, for example benzoic acid, methyl ester; 1,4-benzenedicarboxylic acid, dimethyl ester and hexadecanoic acid, methyl ester appear to increase in both susceptible and tolerant progenies, but more so in tolerant progenies. Meanwhile, 9,12-octadecadienoic acid (Z,Z)-, methyl ester was not present in P1 but

present with relatively lower amount in P3. 9-octadecenoic acid (Z), methyl ester was not present in P1 and P3, the susceptible progenies. 9-octadecenoic acid (Z), methyl ester and hexadecanoic acid, methyl ester from neem oil were reported to express antibacterial activity against three bacterial strains, namely *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* sp. [16]. The level of these metabolite compounds were higher in tolerant progenies and could be acting as potent antimicrobial agents. Octadecanoic acid, methyl ester was much higher in susceptible progenies. This finding is in agreement with Hamzehzarghani *et al*. [9], who reported that certain fatty acids appear to increase at a higher rate in susceptible wheat cultivars, Roblin against *Fusarium* head blight. This cultivar plausibly takes more advantage of the jasmonic acid signal transduction system than Sumai3. The octadecanoic acid pathway produces signal molecules with vital roles in regulating secondary pathways. Benzoic acid was up-regulated in the resistant cultivar Sumai3 after inoculated with a pathogen. Benzoic acid can easily be converted to cinnamic acid, a key compound in the phenylpropanoid pathway. Decarboxylation of trans-cinnamic acid to benzoic acid and further 2-hydroxylation of benzoic acid to salicylic acid have also been reported [9,14]. Some aromatic compounds such as benzoic acid have an important role in signal transduction and were also reported to have antimicrobial activities [8]). The other two phenol compounds found in this study were phenol, 2,6-dimethoxy- and phenol, 2,4-bis(1,1-dimethylethyl). Phenol, 2,4-bis (1,1-dimethylethyl) was absent in all inoculated oil palm progenies; whereas phenol, 2,6-dimethoxy- was increased in P3, P4 and P5 but was slightly lower in P1. Better understanding of the mode of action of these reported metabolite compounds and the mechanisms used by *G. boninense* to outmanoeuvre the oil palm defense system should reveal new possibilities for the directed control of phytoalexin production in specific tissues and at specific developmental stages.

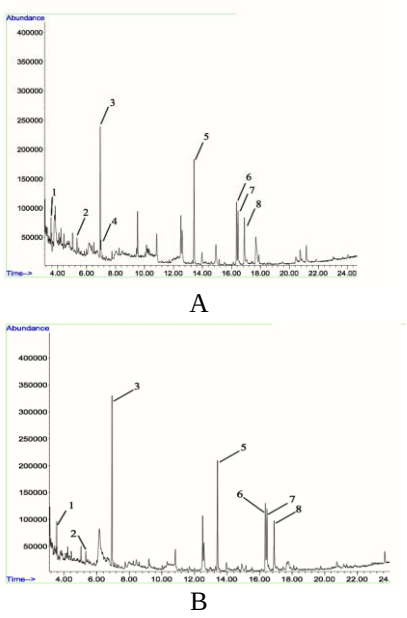

Fig. 2: GC-MS profile of P4 non-inoculated (A) and inoculated (B) oil palm seedlings.

Notes: 1. Benzoic acid, methyl ester
2. Phenol, 2,6-dimethoxy-
3. 1,4-Benzenedicarboxylic acid, dimethyl ester
4. Phenol, 2,4-bis(1,1-dimethylethyl)
5. Hexadecanoic acid, methyl ester
6. 9,12-Octadecadienoic acid (Z,Z)-, methyl ester
7. 9-Octadecenoic acid (Z)-, methyl ester
8. Octadecanoic acid, methyl ester

Conclusion:

The current finding suggests that different oil palm progenies have various levels of resistance towards *G. boninense* based on the disease severity of internal symptoms of bole tissues of the examined oil palm progenies. Metabolite profiling using GC-MS enabled the identification of benzoic acid, methyl ester; hexadecanoic acid, methyl ester; 9,12-octadecadienoic acid (Z,Z)-, methyl ester and 9-octadecenoic acid (Z)-, methyl ester which may play an important role in the oil palm defense system and potentially be used as biomarkers for evaluating oil palm progenies for the development of resistance variety to BSR in the future.

ACKNOWLEDGMENT

We would like to express our deepest gratitude to the Ministry of Education Malaysia for the scholarship given to Rozlianah Fitri Binti Said under the Academic Training Scheme IPTA (SLAI) of Universiti Teknologi MARA and everyone involved. This research was funded by ERGS (ERGS0017-STG-1/2012) and the PhD Incentive Grant (PhD0001-SG-1/2012).

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