Crop Protection 30 (2011) 1438–1442

Contents lists available at ScienceDirect

Crop Protection

journal homepage: www.elsevier.com/locate/cropro

Ergosterol analyses of oil palm seedlings and plants infected with *Ganoderma*

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ARTICLE INFO

Article history:
Received 8 March 2011
Received in revised form
4 July 2011
Accepted 11 July 2011

Keywords:
Ganoderma boninense
Ergosterol
Oil palm
Diagnostic

ABSTRACT

Basal stem rot of oil palm (OP) by *Ganoderma boninense* is of major economic concern and it is the predominant disease of OP in SE Asia. Also, other plantation crops are affected by *Ganoderma*. The early detection of symptoms is crucial for control, although effective methods remain elusive. Ergosterol is the principal sterol of fungi and plays an essential role in the cell membrane and other cellular constituents. The analysis of ergosterol is useful for fungal detection in solid plant substrates. The present report compares ergosterol concentration in sound and decayed OP seedlings and mature plants using HPLC with diode array detection. The disease of OP requires to be considered as a white rot process where fungal biomass will increase from a low to high level as the infection progresses. *G. boninense* biomass was correlated with ergosterol concentration *in vitro*. Furthermore, the sterol was correlated with internal colonization (a) of inoculated seedlings, (b) of felled and standing OP and (c) to external symptoms of the disease. The compound was not detected in healthy samples. Disease treatments may be made more effective as the amounts of fungal biomass can be estimated and early detection is possible. Ergosterol quantification is a provisional diagnostic method for detection for *G. boninense* infection in OP which can be employed with other methods, enabling early remedial action to be taken. The method is recommended for further research involving basal stem rot of OP.

1. Introduction

The major constraint to OP cultivation in Malaysian plantations is the incidence of Basal Stem Rot (BSR) disease caused by *Ganoderma* spp. A similar situation exists in many other countries (e.g. Indonesia) (Ariffin et al.,2000). Furthermore, species of *Ganoderma* are the causal agents of rots of numerous other plantation crops including coconut, rubber, betelnut, tea, cocoa, guarana, and grapevines. Forest trees such as *Acacia*, *Populus* and *Macadamia* are also affected.

The disease is a "white rot" process involving growth of the fungus within the OP as lignin and cellulose are biodegraded (Paterson, 2007a). Detection of BSR is based on the occurrence of unopened OP spear leaves and basidiomata of the fungus appearing on the trunks or primary roots near soil level. Confirmation is by the isolation, growth and identification of the fungus (Lim and Fong, 2005). This is time consuming and requires a high level of taxonomic expertise. The fungus is referred to as *Ganoderma boninense*. At least half of the basal tissue will have been killed by the fungus by the time the foliar symptoms are observed (Ariffin et al.,2000) has been a recurrent theme of previous work.

Paterson (2007a) reviewed the literature on diagnostic methods for *Ganoderma* disease of OP. For example, six chapters in Flood et al. (2000) were devoted to diagnostic related information based on the molecular variability of *Ganoderma* spp. and the development of DNA methods. Paterson (2007b), Paterson et al. (2008) and Paterson and Lima (2009) reviewed PCR based techniques for *Ganoderma* disease of OP. The use of Enzyme-Linked Immunosorbent Assays-Polyclonal Antibody (ELISA-Pab) was reported (Utomo and Niepold, 2000). In addition, the development of (a) PODITOO™ tomography to locate and identify infection and (b) Geographical Information System (GIS) to detect possible sites and distribution of *G. boninense* infection in plantations have been attempted (Idris et al., 2009). Nevertheless, a robust, rapid and reliable early detection method is highly desirable with which to compliment these alternative methods.

Ergosterol is a cell membrane primary sterol specific to fungi (Axelsson et al., 1995) and which has been detected in other *Ganoderma* species (Paterson, 2006). The compound has been used

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doi:10.1016/j.cropro.2011.07.004

to detect fungal invasion in grain and a variety of other substrates such as mycorrhizal roots, wood, foliage, soil and the aquatic environment (Salmanowicz and Nylund, 1988; Gao et al., 1993; Manter et al., 2001; Gong et al., 2001; Gessner and Newell, 2002 and Paterson, 2007c). Furthermore, the use of the sterol to detect growth of other white rot fungi (i.e. *Hydnum* and *Polyporus*) in sterilized OP *in vitro* was reported (Paterson et al.,2000), although the method has not been reported to estimate *Ganoderma* disease within OP. Treatments of *Ganoderma* disease would be made more effective if the amount of infection could be determined and early detection would permit culling of infected OP before the disease spread. Finally, ergosterol complements other methods such as PCR, for detecting plant disease (e.g. Yu et al., 2009).

In the present report, ergosterol was determined in (a) a *G. boninense* isolate from decayed OP, (b) artificially inoculated oil palm seedlings (c) samples of sound and decayed OP stem removed previously from whole OP and (d) whole OP demonstrating disease symptoms in the field.

2. Materials and methods

2.1. HPLC of ergosterol standard

Ergosterol (>95% HPLC pure, Sigma–Aldrich) standards were prepared in methanol and analyzed using reverse-phase HPLC (Supelco). The mobile phase was 100% methanol (HPLC grade) and the column used was an Ascentis express 2.7 μ C18 reverse-phase. The flow rate of the mobile phase was 1.0 ml min^{-1} and the wavelength for the photodiode array detector (Waters Corp.) was 282 nm. The ergosterol retention time was approximately 6.5 min.

2.2. Ergosterol extraction

The *G. boninense* isolate was from a basidiomata of an infected OP trunk which was growing in Banting, Selangor, Malaysia by using *Ganoderma* selective medium (Ariffin and Idris, 1991). The identification was confirmed by a resident plant pathologist, Universiti Putra Malaysia (UPM) and based on spore morphology and cultural characteristic. The pure culture was maintained on potato dextrose agar (PDA) (Merck) and preserved in an in-house culture collection at UPM. *G. boninense* pure cultures were cut to 0.5 cm^2 and inoculated into unshaken 250 ml Erlenmeyer flasks with 100 ml Malt Extract Broth (MEB) (Merck) at ambient temperature (±25 °C) to obtain the mycelium. After 3 weeks, mycelium was removed from the flask, rinsed with sterile distilled water and freeze-dried.

The non-alkaline ergosterol extraction (NAE) protocol used was based on that of Gong et al. (2001) with some modifications in the amount of the glass beads used and time of shaking with glass beads. Mycelium (100 mg) was added to 15 ml polypropylene screw-cap centrifuge tube (Eppendorf) containing 2 g of acid-washed glass beads (750 μm). After the addition of 6 ml methanol, the vial was vortexed for 10 s and shaken intensively for 30 min on a bench-top orbital shaker (Protech) at room temperature, 20–25 °C. The mycelium-methanol mixture was allowed to precipitate for 10 min, and an aliquot of 1.0 ml supernatant was transferred to a 1.5 ml Eppendorf microtube. The microtube was centrifuged for 10 min at 11,000 oscillations min^{-1} in a Sigma Microcentrifugator. The supernatant (1.0 ml) was filtered through a 0.2 μm membrane syringe filter and the filtrate was placed in a 1 ml sample tube that was loaded on an autosampler (Waters Corp.) for HPLC analysis.

2.3. Ergosterol versus fungal biomass

The freeze-dried mycelium was macerated using a mortar and pestle and liquid N_2 until a powder was formed. Different mycelium masses (0.1, 0.5, 1.0 and 2.0 g) in triplicate were extracted for ergosterol. The identification of ergosterol in all samples was achieved by comparing retention times and the diode array UV spectrum against the ergosterol standards.

2.4. Oil palm seedlings

Five month-old OP seedlings were used for the artificial infection study, consisting of two experimental variants (control (T1) and *Ganoderma* infected treatment (T2)). The OP seedlings used were commercial seedlings (Dura x Pisifera) supplied by Sime Darby Research Center at Banting, Selangor which were grown in a glasshouse until they reached the four to five leaf stage. The seedlings were uprooted carefully and transplanted into polybags (size 15 cm × 23 cm) containing 3 kg soil mixture (3:2:1 v/v/v topsoil: peat: sand). Treatments (T2) were inoculated with *G. boninense* colonized rubber wood blocks placed in contact with the roots (Sariah et al., 1994). Uninoculated seedlings were used as negative control. All OP seedlings were placed and arranged in complete randomized blocks under glasshouse conditions for 22 weeks. Destructive sampling of the seedlings was done over a period of 3, 6, 10, 14, 18 and 22 weeks to assess BSR infection. Random root samples (3 g) were weighed and powdered finely with a mortar, pestle and liquid nitrogen. Samples were subjected to extraction followed by HPLC analysis. Ten inoculated and ten uninoculated seedlings were harvested for each sampling time. A visual assessment of BSR infection was determined by examining the roots of the seedlings and the severity of the symptoms expressed based on the proportion of lesioned (rotting) root.

2.5. Oil palm sections

Fifteen diseased and 3 healthy OP standing trunks were cut down with a chain saw from BSR-infected areas in the Malaysian Palm Oil Board (MPOB) Experimental Station Kluang, Johor. The palms were cut approximately 0.5–1 m from the base of the trunk and the BSR disease internal symptoms were observed by examining the cross section of cut OP stem tissue. The samples were classified visually from zero (healthy palm), low (yellow to light brown infected tissue), medium (brown infected tissue without fermented rancid odor) and high rot (extensive layer of dark brown infected tissue with strong fermented rancid odor). This is a standard method as described by Ariffin et al. (2000). The healthy and infected cut OP stem samples were slice into small blocks (12 cm × 12 cm) with the chain saw and transported to the laboratory in an ice-chest and stored at −20 °C. The block samples were ground using a Waring laboratory blender into a powder and tissue samples of 3 g were further ground using a mortar and pestle with liquid nitrogen to produce a fine powder. Samples were subjected to extraction followed by HPLC analysis.

2.6. Standing oil palm

Mature OP tissue samples were collected from high BSR disease incidence areas situated at Sime Darby Research Center, Banting, Selangor. The OP samples were chosen based on the appearances of external symptoms of BSR disease on the palm and were categorized into healthy, low infection and severe infection palms. Ten palms were sampled for each category. A Husqvarna 61 driller with drill size diameter 19 mm and length 15 cm (from MPOB) was used to penetrate the OP trunk to 8–20 cm of depth from four points

(north, west, east and south). Each drill point was approximately 1 m from the base of the palm. A total of 15–20 g of tissue samples was collected and pooled from each palm and placed into a clean container, which was transported to the laboratory in an ice-chest and stored at−20 °C. Ergosterol was extracted using the NAE method. Tissue samples (3 g) were weighed and powdered finely with a mortar, pestle and liquid nitrogen. Samples were subjected to extraction followed by HPLC analysis.

2.7. Statistical analysis

Statistical analysis was performed using SAS (version 9) software. The significant differences between means were determined using one-way analysis of variance (ANOVA) at 95% confidence level. Products with the same letter have no significant difference with $P > 0.05$. Correlation analysis was performed using Microsoft Excel 2007.

3. Results

The HPLC responses (peak area) were check for linearity within levels (0.1–200 μg ergosterol standard) for ergosterol standards. They were correlated with ergosterol concentrations and gave good correlation coefficients (R^2) of 0.9982. Ergosterol was detected from the *G. boninense* isolate and the concentration increased directly with the increase of biomass (Fig. 1). The lowest and highest mycelial biomass of 0.1 g and 2.0 g resulted in the lowest and highest ergosterol concentrations of 30.6 μg g^{-1} and 170.3 μg g^{-1} respectively.

The ergosterol concentrations increased significantly with the increase in the degree of root infection from 3, 6, 10, 14, 18 and 22 weeks after inoculation in the *Ganoderma* inoculated seedlings (Fig. 2). Ergosterol was absent in all uninoculated seedlings. The (a) highest concentration of ergosterol was 10.34 μg/g on week 22 with >50% of root infection and (b) lowest concentration was 0.04 μg/g in week 3 with less than 10% infection of the roots infected. A good correlation ($R_f = 0.959$) was observed between the percentages of root infection and the ergosterol concentration after 3, 6, 10, 14, 18, and 22 weeks.

The sensitivity of ergosterol as a marker for detection of *G. boninense* in BSR-infected palms is indicated in Table 1 with respect to the OP section method. Ergosterol was detected in all infected OP samples (infected 1–15) and was absent in healthy samples (healthy 1–3). Ergosterol concentrations varied from each infected palms ranging from 0 to 50.0 μg g^{-1}, which is equivalent to 0.01–0.20 g *G. boninense* g^{-1} OP tissue as deduced from the standard curve. The average ergosterol concentrations for healthy, low, medium and highly-infected OP were 0.00, 3.90, 16.11, and 29.72 μg g^{-1} OP respectively (Fig. 3).

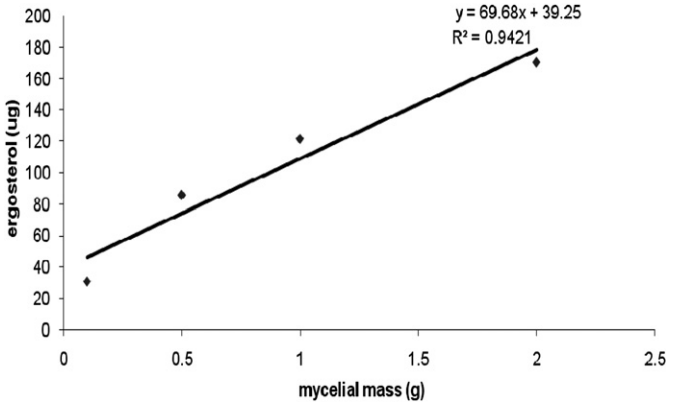

Fig. 1. Quantification of ergosterol in different weights of *G. boninense* mycelia. Data represent means and standard deviation of three replicated samples.

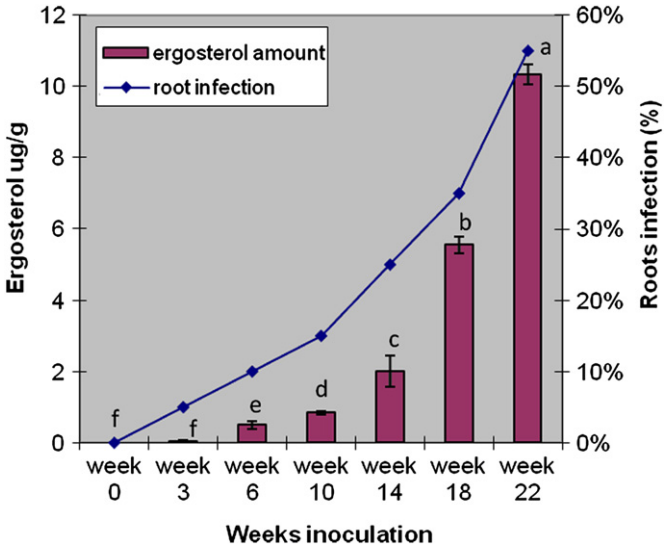

Fig. 2. Ergosterol concentration and root infection percentage in inoculated seedlings. Bars represent means ± SD (error bar) of 10 replicated samples. Significant different for each disease level indicated by different letters ($P < 0.05$, ANOVA).

Ergosterol was detected in all infected OP samples using the standing OP method and absent in all healthy palm samples. Ergosterol concentration varied from (a) slightly-infected palms (low infection 1–10) with concentrations from 0.85 to 5.02 μg g^{-1} OP tissue to (b) severe infection samples 1–10 with concentrations from 17.27 to 42.56 μg g^{-1} OP tissue. The relationship of ergosterol concentration with external symptoms of BSR is indicated in Table 2. The mean ergosterol concentrations for healthy, low and severely-infected OP were 0.00, 2.56, and 26.22 μg g^{-1} OP respectively (Fig. 4).

Table 1
Relationship between ergosterol concentration and organoleptic assessment (OA) of disease severity from infected oil palm section samples. Ergosterol data represent standard deviation of three samples per palm.

Oil Palm	[a]OA of BSR disease severity	Ergosterol concentration (ug/g)
Healthy 1	0	not detected
Healthy 2	0	not detected
Healthy 3	0	not detected
Infected 1	Low	0.93 ± 0.16
Infected 2	Low	2.32 ± 0.14
Infected 3	Low	4.34 ± 0.24
Infected 4	Low	4.66 ± 0.16
Infected 5	Low	7.27 ± 0.19
Mean		**3.90 ± 2.42**
Infected 6	Medium	15.74 ± 0.23
Infected 7	Medium	15.34 ± 0.23
Infected 8	Medium	17.24 ± 0.26
Mean		**16.11 ± 1.00**
Infected 9	High	17.33 ± 0.24
Infected 10	High	18.45 ± 0.13
Infected 11	High	23.56 ± 0.25
Infected 12	High	23.85 ± 0.19
Infected 13	High	37.60 ± 0.23
Infected 14	High	38.65 ± 0.41
Infected 15	High	48.60 ± 0.34
Mean		**29.72 ± 11.91**

low–Yellow to light brown infected tissue medium–Brown infected tissue without fermented rancid odor high–Extensive layer of dark brown infected tissue with strong fermented rancid odor.

[a] Disease severity score.

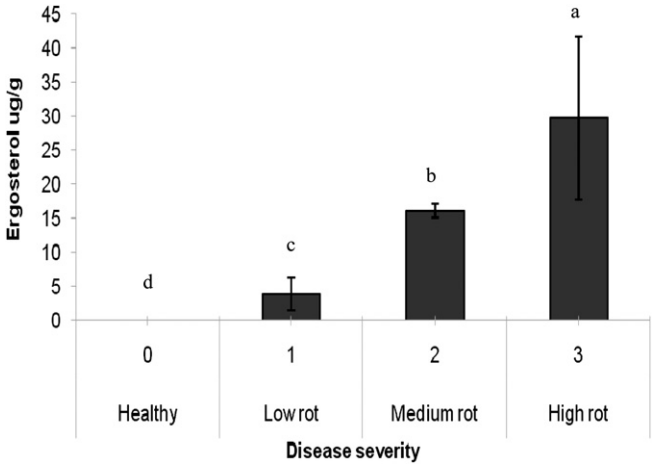

Fig. 3. Mean ergosterol (μg g^{-1} OP tissue) compared to degree of rot of oil palm sections. (0 = healthy tissue; 1 = low rot as assessed by a yellow to light brown infected tissue; 2 = medium rot as assessed by brown infected tissue without a fermented rancid odor; 3 = high rot as assessed by an extensive layer of dark brown infected tissue with a strong fermented rancid odor). Bars represent means ± SD (error bar) of three samples per palm. Significant different for each disease severity indicated by different letters ($P < 0.05$, ANOVA). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

The present paper represents the first report for detection of ergosterol from *G. boninense*, although the compound has been detected from other species of *Ganoderma* (Paterson, 2006). The current work demonstrates uniquely that the sterol can be employed to quantify and detect the BSR symptoms in OP. Paterson et al. (2000) had established that ergosterol could be employed to measure growth of *Hydnum* and *Polyporus* in an *in vitro* system using OP blocks.

The relationship between ergosterol concentration and *G. boninense* mycelial biomass indicated that ergosterol increased proportionally to biomass. Similar findings also have been reported from other fungi (Paterson et al.,2000; Manter et al., 2001; Ng et al. 2007; Paterson 2007c). These findings suggested that ergosterol related directly to the growth and severity of *G. boninense* infection in OP grown in plantations. Artificially inoculated seedlings demonstrated an increase in ergosterol concentration with greater root infection over time but it was not detected in uninoculated seedlings. This further indicated the effectiveness of ergosterol for monitoring *G. boninense* infection. Furthermore, ergosterol was not detected in healthy palms, while it was present in all infected OP samples and the concentration varied proportionately to the degree of infection. A direct relationship between internal symptoms and ergosterol concentrations was demonstrated. Furthermore, ergosterol concentration was related directly with the external symptoms of BSR and indicated that the method is suitable for direct sampling in the field.

It may be worth pointing out that it is extremely difficult to undertake numerous repeats within the same sets of experiments as might be possible on other crops, due to the nature of the host. OP is very slow growing and normally takes 20 years to reach maturity. We compensated for this limitation by using 15 palms per analysis which is a high level of repetitions for OP-related experimentation.

Ergosterol is a robust method for detection for *G. boninense* infection in oil palm. The fungal metabolite is absent from vascular plants and most other organisms, although it may be present in a minor amounts, in bacteria, cyanobacteria, green and microalgae and protozoa (Gessner and Newell, 2002). In addition, plants produce different sterols such as sitosterol and phytosterols which

Table 2
Relationship between ergosterol concentration and external symptoms assessment of disease severity from infected standing oil palm stem samples. Ergosterol data represent standard deviation of three samples per palm.

Samples[a]	Ergosterol concentration[b]
Healthy	
1	ND[c]
2	ND
3	ND
4	ND
5	ND
6	ND
7	ND
8	ND
9	ND
10	ND
Mean	**ND**
Low	
1	2.32 ± 0.34
2	4.58 ± 0.30
3	3.93 ± 0.14
4	0.93 ± 0.16
5	2.52 ± 0.24
6	0.85 ± 0.18
7	5.02 ± 0.16
8	1.53 ± 0.26
9	1.10 ± 0.17
10	2.85 ± 0.18
Mean	**2.56 ± 1.52**
Severe	
1	17.27 ± 0.20
2	24.66 ± 0.18
3	18.33 ± 0.21
4	25.34 ± 0.17
5	23.85 ± 0.18
6	27.24 ± 0.31
7	24.74 ± 0.25
8	19.56 ± 0.17
9	38.65 ± 0.41
10	42.56 ± 0.22
Mean	**26.22 ± 8.30**

[a] Samples (disease severity score). Low—appearance of foliar symptoms (1–2 unopened spear leaves). Severe—appearance severe foliar symptoms with or without appearance of fruiting body.
[b] Ergosterol data represent mean and standard deviation of three samples per palm.
[c] ND = no data.

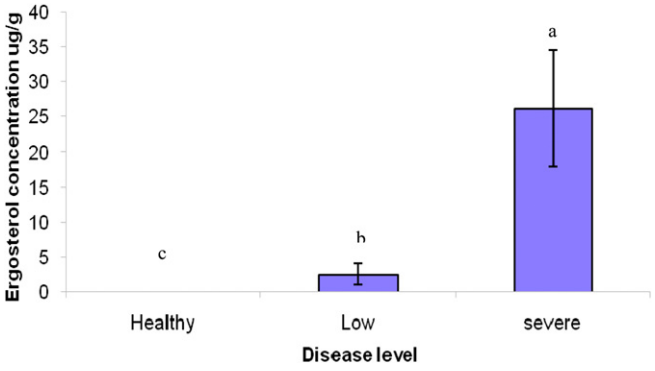

Fig. 4. Mean ergosterol (μg g^{-1} OP tissue) compared to degree of BSR external symptom in standing oil palm. (Healthy = healthy palms; Low = low infection assessed by appearance of foliar symptoms (1–2 unopened spear leaves); Severe = severe infection assessed by severe foliar symptoms with or without appearance of fruiting body). Bars represent means ± SD (error bar) of three samples per palm. Significant different for each disease level indicated by different letters ($P < 0.05$, ANOVA).

are not produced in fungi (Weete, 1974). These results demonstrate that ergosterol concentration can be used to assess the growth of the fungus within the palm and may be useful for root, foliar and other samples (Salmanowicz and Nylund, 1988; Manter et al., 2001; Gong et al., 2001).

In conclusion, the results from our studies support the view that ergosterol is a diagnostic method with utility for the detection of BSR in oil palm. This represents the first data published on the use of ergosterol analysis as a diagnostic method to detect BSR and indicates a new direction of research in BSR management.

Acknowledgements

The authors wished to thank the Malaysian Palm Oil Board (MPOB) for the supply of materials in this research and Universiti Putra Malaysia (UPM) under the Fundamental Research Grant Scheme for the research grant received. R.R.M. Paterson gratefully appreciates obtaining the IOI Professorial Chair, Department of Plant Protection, Universiti Putra Malaysia, Malaysia under which this work was initiated, and for the FCT framework position: Commitment to Science ref. C2008-UMIN-HO-CEB-2.

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