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Electronic Nose detection of *Ganoderma boninense* Volatile Organic Compounds (VOCs) Using Direct Headspace Analysis

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ABSTRACT Electronic nose detection techniques have found wide applications in many fields including the medical and health sector, food industry, and also agriculture. Detection of volatile organic compounds (VOCs) emitted by *Ganoderma boninense* is increasingly explored as a reliable means of accurately diagnosing the presence of this important oil palm pathogen, facilitating management of the disease and preventing further spread in the field. For this purpose, we tested an electronic nose ('zNOSE™ 4200') to detect *G. boninense* VOCs via direct headspace analysis from samples of trunk cores and inoculated oil palm ramets. We successfully identified five VOC markers (two for healthy tissue, three associated with *G. boninense* infection). Based on gas chromatography-mass spectrometry (GC-MS) of samples and analytical standards, one of the VOC markers for *G. boninense*-infected palms was positively identified as 2, 4-di-tert-butylphenol. These markers can be used in the next stage of optimization and field testing.

KEYWORDS: Volatile organic compounds; direct headspace; early detection; basal stem rot; *Ganoderma boninense*
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Full Article

INTRODUCTION

The Basidiomycete fungus *Ganoderma boninense* is a major factor constraining the productivity of both small- and large-scale plantations of the African oil palm (*Elaeis guineensis*) in Southeast Asia. *G. boninense* causes basal stem rot (BSR) and / or upper stem rot (USR) symptoms, but BSR is more common and USR is more often observed in parts of Borneo. Despite its long history in oil palm plantations, the complete lifecycle and pathogenesis are still not fully understood, which complicates disease control on the ground. Currently, the conventional approach in most estates is largely confined to removal of diseased tissues and preventing spread to healthy palms by mechanically chipping and / or pulverizing the oil palm trunk and bole during replanting. Ideally, infected palms should be eradicated as soon as possible, but due to the slow progression of the disease, these palms are often undetected until it is too late for remedial action. Thus, the key is to identify infected palms even before visual symptoms are observed.

In recent years, government, academic as well as industry players have investigated the usefulness of various technologies to qualitatively and quantitatively detect *Ganoderma*, which include but are not limited to sonic tomography (Ishaq *et al*., 2014), hyperspectral remote sensing (Shafri *et al*., 2011; Izzuddin *et al*., 2018), carbon nanotube (Isha *et al*., 2019) and electronic nose (Zainol Hilmi *et al*., 2019). From our previous evaluation, some of these technologies, although highly accurate, may be too costly, cumbersome or slow to be used for large-scale commercial testing of individual palms. Ideally, the technology for detection should be non-invasive, allows high accuracy and precision of early detection, easy to use and inexpensive. Electronic noses have

been widely applied in various fields such as human disease diagnosis (Turner & Magan, 2004; Sánchez *et al*., 2019), determination of food freshness and quality control (Benedetti *et al*., 2004; Mohamed *et al*., 2018), and also in plant pathogen recognition (Ghaffari *et al*., 2012; Cellini *et al*., 2017; Wilson, 2018). However, there are numerous approaches and challenges with optimizing the electronic nose for its desired purpose. Since 2017, we have collaborated with Agri-Diagnostics Sdn Bhd to test the use of an electronic nose ('zNOSE™ 4200') for detecting volatile organic compounds (VOCs) diagnostic to *G. boninense* or the diseased tissue. We have evaluated zNOSE™ 4200 on inoculated oil palm trunk tissue and inoculated oil palm clonal plantlets *in-vitro*. This project is ongoing and we are glad to share some of the promising results prior to field-testing.

BACKGROUND THEORY

How the z-NOSE™ 4200 works

z-NOSE™ 4200 was supplied by Electronic Sensor Technology (Newbury Park, California, USA) and consists of two parts. The first uses helium gas, a capillary tube (DB-5 non-polar gas chromatography column, Agilent) and a solid-state detector. The second part comprises a heated inlet and a pump which samples ambient air. Before use, the equipment is calibrated using a single n-alkane vapour standard. Samples are placed into capped GC vials. Air samples drawn in are first pre-concentrated in a 'loop' trap situated between the aforementioned two parts, mixed with the helium and injected into the DB-5 column. After exiting the column, individual chemical compounds are deposited onto a sound acoustic wave (SAW) detector, which consists of an uncoated quartz crystal. Change in the oscillating frequency of the resonator reflects the molecular mass of the compounds. Data collected by the sensor is subsequently transferred to a user interface. A library of retention times of known chemicals is indexed to the n-alkane response (Kovats indices), which allows for machine independent measurement and compound identification. The time derivative of the sensor spectrum yields the spectrum of column flux (chromatogram).

METHODOLOGY

Inoculum source and maintenance

A single pathogenic and aggressive isolate of *G. boninense* (T10) was used in this experiment (Kok *et al*., 2013). The *G. boninense* inoculum was prepared and maintained as per Kok *et al.* (2013).

Trunk core sampling and inoculation

Figure 1. Trunk cores were removed from a chunk of healthy mature oil palm trunk (left) via an increment borer (middle), which were then placed into GC vials with or without *G. boninense* inoculum (right, inoculated vials shown).

A visually healthy (asymptomatic) palm was selected from a mature oil palm field planted in 1997 located in Jeram, Selangor (GPS coordinates 3.190870, 101.356412). The palm was felled with a

chainsaw and checked to ensure absence of internal rot. A portion of the healthy trunk (around 40cm x 40cm x 40cm) furthest away from the roots was excised and brought back to the lab (**Figure 1**). After surface-sterilization, three trunk cores, each around 5cm in length, were made with a forestry increment borer and placed into clean glass 40ml GC vials (Agilent 5183-4334) fitted with PTFE / silicone septa caps (Agilent 5183-4308). Twenty vials of the trunk cores were designated as controls and twenty vials were designated in the treatment group. Each of the treatment vials were added with three 12mm-diameter discs of *G. boninense* isolate T10 grown on solid malt extract agar (MEA). After capping, all the vials were incubated in darkness at 26 ± 2 °C (**Figure 1**). Readings with the zNOSE™ were taken at 14 days post-inoculation (**Figure 2**).

Figure 2. zNOSE™ equipment (left) and the method of reading the headspace VOCs (right).

In-vitro *inoculation*

Table 1. Design of the *in-vitro* ramet inoculation experiment

Treatment	Days post-inoculation (DPI)	Replicates
MS media only	1	1
MS media + *G. boninense* T10	1	3
	23	3
MS media + oil palm ramet	1	3
	23	3
MS media + oil palm ramet + *G. boninense* T10	1	3
	23	3
Total		19

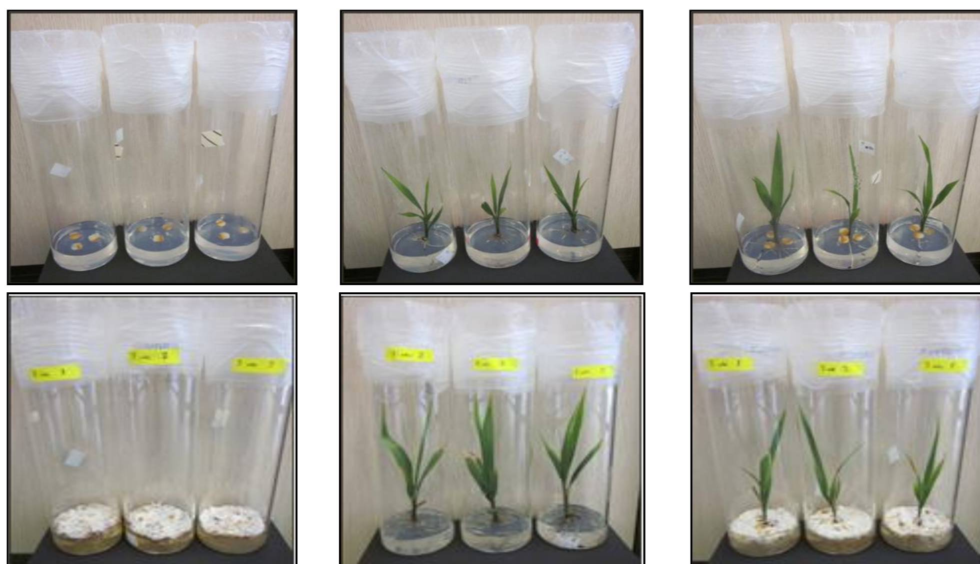

Figure 3. The glass containers with *G. boninense* isolate T10 only (left column), oil palm ramets only (middle column) and oil palm ramets with T10 (right column) at 1 day post-inoculation (top row) and at 6 weeks post-inoculation (bottom row). zNOSE™ 4200 readings were taken at 23 DPI.

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Nineteen large clean glass containers (30cm high, 7cm diameter) were filled up to around 3cm high with Murashige-Skoog (MS) plant tissue culture media. The containers were covered with aluminium foil and autoclaved as per Kok *et al.* (2013). Twelve sterile oil palm tissue culture plantlets / ramets (same clone) were obtained from the AAR tissue culture laboratory and one ramet was transferred into each of the glass containers as detailed in **Table 1**. In the remaining glass containers, three 12-mm diameter discs of *G. boninense* isolate T10 on malt extract agar (MEA) were introduced as needed, following **Table 1**. The containers were capped with food-grade silicone covers held in place with Parafilm. All of the containers were placed under artificial lighting at 16-hour day / 8-hour dark cycles at 26 ± 2 °C (**Figure 3**). Readings with the zNOSE™ were taken at 1 and 23 days post-inoculation in the same manner as shown in **Figure 2**.

Identification of putative G. boninense *VOCs*

The results generated by zNOSE™ were compiled and compared to publicly-available VOC databases (MVOC 2.0, Odour.org) on the internet and existing literature. After identifying several putative *G. boninense* VOCs, these were confirmed by purchasing analytical-grade chemical standards (Sigma) of the said chemicals and running them through zNOSE™ as previously described. The chemical standards and several of the inoculated trunk core samples were also sent for gas chromatography-mass spectrometry (GC-MS) at Universiti Putra Malaysia (UPM). Comparisons of peaks and retention times enable us to ascertain whether the specific VOC is diagnostic of the presence of *Ganoderma* only, healthy tissues only, or interaction (rot or infection).

RESULTS AND DISCUSSION

In the trunk core samples (**Figure 4**), five of the VOCs detected via zNOSE™ were unique to healthy oil palm tissue, and eight VOCs were detectable only when *G. boninense* was present. However, as the uninoculated samples would also be decaying naturally within the 14-day incubation period, it is likely that some of the VOCs emitted are released as a result of organic matter decomposition by enzymes and endophytic microflora. A wider range of VOCs was detected from the *in-vitro* ramet samples (**Figure 5**). There was slight variation between replicates with regards to specific VOCs emitted and amount detectable but the VOC composition also changed with incubation time, reflecting progression of pathogen growth on the oil palm ramets. This setup allowed us to eliminate the possible confounding of decay-related VOCs in the control group, as all the uninoculated ramets remained alive and healthy until the end of the experiment at 6 weeks post-inoculation (**Figure 3**). We confirmed the presence of several plant VOCs from the literature (Babushok *et al.*, 2011, AAR data, unpublished) and narrowed down *Ganoderma*-related VOCs using the publicly-available MVOC 2.0 database. Although data on *G. boninense per se* was not available on the database, species in the same genus (*G. adspersum, G. applanatum, G. lucidum*) are also plant pathogens on certain woody trees (non-palms) and share similar pathogenesis pathways.

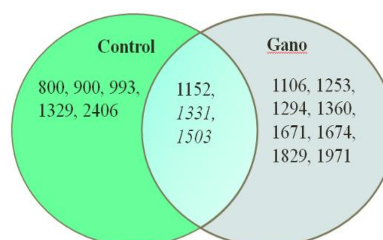

Figure 4. Venn diagram of various chemical compounds detected from the trunk core samples via zNOSE™ 4200 at 14 days post-inoculation (DPI). The numbers are the Kovats indices (KI), each

corresponding to a specific molecular mass. Italicized KI numbers indicate chemicals present in both sample groups but at much higher concentration in *G.boninense*-inoculated trunk cores.

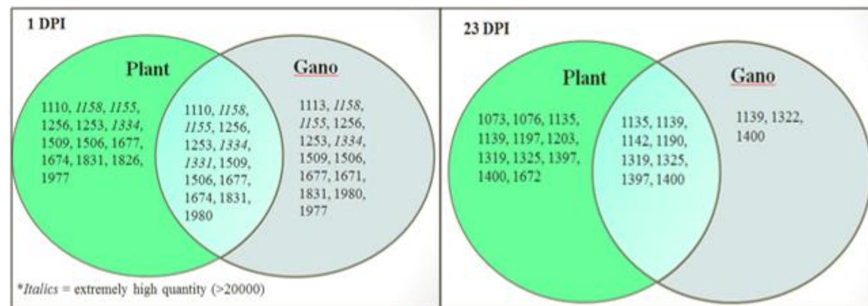

Figure 5. Venn diagram of various chemical compounds detected from the *in-vitro* oil palm ramet samples via zNOSE™ 4200 at 1 and 23 days post-inoculation (DPI). The numbers are the Kovats indices (KI), each corresponding to a specific molecular mass. Italicized KI numbers indicate chemicals present in both sample groups but at much higher concentration in inoculated samples.

As shown in **Figure 6**, the GC-MS results of the trunk core samples clearly show higher number of distinct peaks in the inoculated samples compared to uninoculated trunk cores. Testing of seven chemical standards to confirm the putative identity (2-methyl-1-propanol, 3-methylbutanol, 2, 4-di-tert-butylphenol, 2, 6-di-tert-butylphenol, phenylacetaldehyde, butyric acid and alpha-terpinyl acetate) resulted in correct identification of 2, 4-di-tert-butylphenol (Kovats index of 1520) as present at very high concentrations only in *Ganoderma*-inoculated samples and it is undetectable in healthy trunk cores. Two other unidentified VOC markers (Kovats indices of 1350 and 1460) were also detected only in *G. boninense*-inoculated trunk cores but at lower concentrations. Conversely, two other unidentified VOCs (Kovats indices of 1500 and 1670) were found present only in uninoculated trunk cores and only in a very small number of the inoculated samples, indicating that these could be components which have been rapidly metabolised by *G. boninense* as it infects and degrades the oil palm tissue. These five VOC markers (two for healthy, three for infected) will be subsequently used in the next step of optimizing zNOSE™ 4200 for field testing.

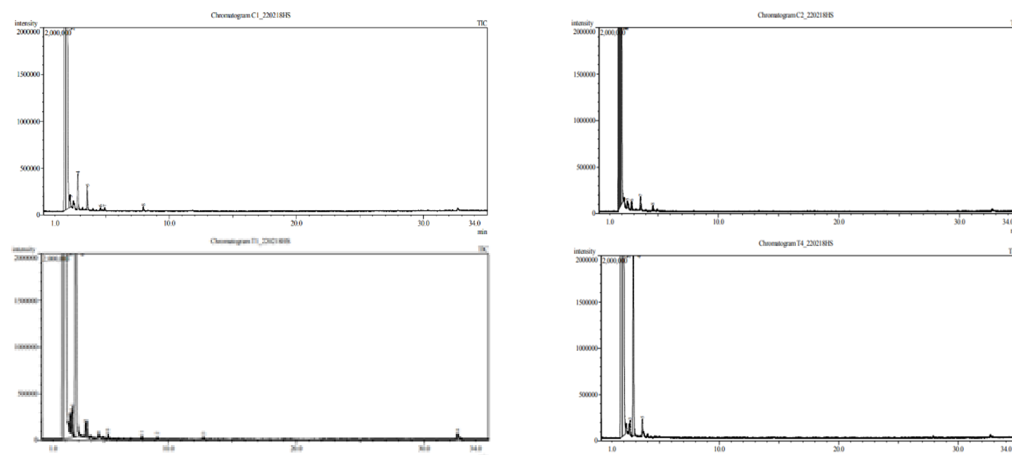

Figure 6. GC-MS chromatogram of two representative healthy trunk core samples (top row) and two representative *G. boninense*-inoculated trunk core samples (bottom row).

CONCLUSION

zNOSE™ 4200 has proven to be an accurate and quick tool for rapid detection of VOCs associated with *G. boninense* infection. Optimization of VOC markers and non-invasive sampling procedures in the field are required before commercial field-testing can be conducted.

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