Journal of Oil Palm Research
DOI: https://doi.org/10.21894/jopr.2023.0065

ANTIFUNGAL VOLATILE ORGANIC COMPOUNDS (VOC) FROM A STRAIN OF *Fusarium foetens* AGAINST *Ganoderma boninense*

ANISA LUTFIA[1]* and BEDAH RUPAEDAH[1]

ABSTRACT

Basal Stem Rot (BSR) caused by Ganoderma boninense *poses a threat to the sustainability of oil palm (*Elaeis guineensis *Jacq.), a valuable export commodity in Southeast Asia. This study focuses on evaluating the potential of an indigenous strain of* Fusarium*, as a biocontrol agent for BSR. The research involves chemical characterisation and laboratory testing. Molecular identification confirmed the isolate as* Fusarium foetens*, an unconventional biocontrol agent that showed promising results in experimental settings. In the disc vapour assay, discs containing volatile organic compounds (VOCs) effectively inhibited the growth of* G. boninense *by up to 60%. Analysis of scanning electron microscopy (SEM) imagery suggested that the inhibition might be attributed to cell wall damage and internal disruption caused by the VOCs. Four major VOCs were identified and ranked based on their relative peak area (%), including 2,3-Pyrazinedicarboximide (15.54%), 2-(2-Butoxyethoxy) ethyl thiocyanate (11.43%), 3,5-Dimethoxyphenol (11.40%), and Indolylmethylthiohydroximate (5.56%), with other minor compounds accounting for less than 5.00%. Docking simulations using PyRx software were performed to analyse the binding affinity between selected VOCs and the virulent protein, xyloglucan-specific endo-beta-1,4-glucanase (XEG). Interestingly, two compounds, 2,3-Pyrazinedicarboximide and indolylmethylthiohydroximate, had exhibited binding affinities similar to that of hexaconazole, a standard antifungal agent used in BSR management.*

Keywords: antifungi, basal stem rot, *Fusarium*, molecular docking, xyloglucanase.

Received: 26 June 2023**; Accepted:** 30 October 2023**; Published online:** 27 December 2023.

INTRODUCTION

Plant pathogens, also known as phytopathogens, pose a major risk to both agriculture and global food security by inducing various plant diseases that result in significant economic losses. The oil palm (*Elaeis guineensis* Jacq.), a valuable export commodity in Southeast Asia, particularly in Indonesia and Malaysia, faces threats to its production and sustainability, due to basal stem rot (BSR), predominantly caused by *Ganoderma boninense* Pat., a basidiomycete fungi. Despite ongoing research efforts, the exact infection mechanism of *G. boninense* in oil palm plantations and its sporadic spread still remains elusive (Bharudin *et al*., 2022).

The management of BSR presents significant difficulties and challenges due to the infective dikaryotic mycelium that arises from the mating between mycelia and newly germinated basidiospores (Pilotti *et al*., 2003; 2018). To date, an effective and integrated disease management method to prevent the spread of *G. boninense* and sustainably reduce the incidence of the disease has not been discovered (Siddiqui *et al*., 2021). Various methods have been employed to control the spread of infection, including soil mounding, manual removal of infected tissues and *G. boninense* fruiting bodies, as well as the use of fungicides such as hexaconazole (Hushiarian *et al*., 2013; Idris *et al*., 2010). Hexaconazole has been considered relatively

[1] Research Center for Applied Microbiology, National Research and Innovation Agency, Jl. Raya Jakarta-Bogor Km. 46, Cibinong, Bogor, West Java 16911, Indonesia.

* *Corresponding author e-mail: anis031@brin.go.id*

effective in reducing the incidence of *G. boninense* infection and infestation in healthy, mature oil palm trees (Khoo and Chong, 2023; Nur-Rashyeda *et al.*, 2023). However, its efficacy decreases when faced with melanised mycelium, basidiospores, and pseudosclerotia of *G. boninense* (Susanto *et al.*, 2005). The continuous use of hexaconazole also carries specific risks, particularly concerning the accumulation of residues on oil palm leaves, detectable for at least 70 days after application (Muhamad *et al.*, 2012).

Various groups of microorganisms, including actinomycetes, bacteria, and fungi, have been investigated as potential biological control agents for *G. boninense*. However, their efficacy is still being assessed, both as individual isolates and consortia, in laboratory and field-scale experiments (Bharudin *et al.*, 2022; Shariffah *et al.*, 2015; 2020). This study aims to assess the effectiveness of a soil fungus belonging to the unique group of *Fusarium*, through both *in vitro* and *in silico* experiments. In addition, the volatile organic compounds (VOCs) produced by this fungus and its effects will be investigated against the *G. boninense* colony. VOCs are a blend of volatile metabolites produced by both microbial and plant sources, which is referred to as "volatilome". They are distinguished by functional effects in the soil, greater ability to disperse, and stronger antifungal properties (Tilocca *et al.*, 2020). Through this research, novel opportunities are sought for harnessing non-pathogenic strains of soil microbes, as potential candidates in the development of antifungal agents.

MATERIALS AND METHODS

Biological Materials and Molecular Identification

The soil fungus, *Fusarium* sp. isolate F2, was obtained from healthy and non-infected oil palm plantation soils in Bogor, Indonesia. Soil samples were serially diluted to 10^8 dilutions. Fungal isolation was carried out using Potato Dextrose Agar (PDA), followed by incubation at 25°C for two to three days (Ali *et al.*, 2016). The pathogenic fungus, *G. boninense* strain SSU008, utilised in this study, was collected from the Oil Palm Research Center (PPKS) Marihat, Simalugun Regency, North Sumatra, Indonesia. The isolates were maintained on PDA medium. To determine their molecular identity, the fungal specimen (isolate F2) was commercially sent to Macrogen, Inc. (Singapore). The genomic DNA was amplified using a pair of ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primer, assembled, and analysed for similarity against an online database using BLASTn for the ITS-rDNA region (Xie *et al.*, 2008). The fungal species were identified by constructing a phylogenetic tree or dendrogram, which displayed the species phylogeny and clustering analysis among accessions using MEGA11 (Tamura *et al.*, 2021). The confirmed species was then submitted as an online accession to GenBank and assigned the accession code *Fusarium foetens* F2 (OR186217).

VOCs Production of *Fusarium foetens* F2 in Submerged Fermentation

For the production of VOCs, potato dextrose broth (PDB) was employed as the fermentation medium (Vieira *et al.*, 2008). The fungus, *F. foetens* F2, was cultivated in a 250 mL flask containing 50 mL of PDB at a temperature of 28°C for 14 days. To obtain the cell-free supernatant (CFS), the fermentation broth was filtered using Whatman filter paper No. 1 in a Büchner funnel. Subsequently, the CFS was separated by centrifugation at 10 000×g for 15 min. The resulting CFS was subjected to extraction using laboratory-grade ethyl acetate (EtOAc) in a 1:1 ratio. The mixture was vigorously shaken for 3 days. The ethyl acetate layer was then separated and decanted, followed by evaporation *in vacuo* to concentrate the crude VOC-containing extract and to remove EtOAc solvent using a Buchi Rotavapor® R-300.

Antifungal Vapour Assay of VOCs and Microscopy Analysis of *G. boninense*

To evaluate the antifungal activity of the VOCs produced by the fungus, *F. foetens* F2 against *G. boninense*, a modified disc diffusion or vapour assay was conducted (Bismarck *et al.*, 2020). An agar plug (ø6 mm) of the 5-day-old colony of *G. boninense* was inoculated and placed at the centre of a potato dextrose agar (PDA) medium. In the lid of the agar plate, a disc containing an extract of ethyl acetate (EtOAc) or saturated VOCs was positioned. The plates were then incubated for five days at 28°C, with the lids facing upwards. A control plate consisting solely of the *G. boninense* colony in the centre was also prepared. The assay was performed in triplicate. The percentage of radial growth inhibition (%) was calculated using Equation (1):

$$(\%) = [(D1 - D2) / D1] \times 100\%, \quad (1)$$

D1 represents the radial growth (mm) of the control plate, and D2 represents the radial growth of the treated plates. For analysis of the treated colony or mycelium, the surface morphology was examined using a scanning electron microscope (JSM-6510LA JEOL SEM). The slide was fixed and coated with platinum (35 s: 30 mA) using 10 kV.

GC-MS Profiling of VOCs

Qualitative analysis of the crude VOC-containing extracts was performed using an Agilent column (Type 19091S-433: 93.92873 DB-5MS UI, 5% Phenyl Methyl Silox) with dimensions of 30 m × 250 μm × 0.25 μm. The temperature range for injection was set from 0°C to 325°C, with a final hold time of 1 min. The analysis was conducted on an Agilent-type 7890 gas chromatograph (GC) coupled with a 5977A mass selective detector (MSD).

In Silico Molecular Docking

Prediction of binding sites of selected VOCs produced by *F. foetens* F2 and their affinities, to xyloglucan-specific endo-beta-1,4-glucanase (XEG) (PDB ID: 3VL8) was performed through a molecular docking analysis (Yoshizawa *et al.*, 2012). The water molecules were removed from the protein using the BIOVIA Discovery Studio 2021 Client 21.1.0 software. The PyRx ver. 0.8 software (Dallakyan and Olson, 2015) was used for the docking simulation. The three-dimensional (3D) conformers of the VOCs were obtained from PubChem site (Bethesda, MD, USA). The results of molecular docking were visualised and analysed for binding affinity/energy (ΔG, kcal/mol) and similarity of binding site residues.

RESULTS AND DISCUSSION

Fusarium sp. F2, a soil fungus thriving in a local oil palm plantation, was successfully isolated. Its presence in asymptomatic regions free from *G. boninense* infection and BSR led to the hypothesis that it may possess antagonistic traits.

In previous studies, researchers have also isolated microbial strains and isolates that demonstrated antagonistic effects against the same pathogenic fungus. These strains were obtained either from the rhizosphere or as endophytes residing in healthy areas (Pramudito *et al.*, 2020; Yurnaliza *et al.*, 2014). Based on the bioinformatics analysis and phylogenetic inference within the ITS-rDNA region, the isolate was then identified as *Fusarium foetens* F2 (*Figure 1*). Some studies have well documented this species as a pathogenic fungus affecting *Begonia elatior* and its hybrids and also potato plants, resulting in plant wilting and abnormal growth (Liu *et al.*, 2023; Tschöpe *et al.*, 2007). However, the presence of *F. foetens* in the oil palm plantation poses an intriguing question that demands further investigation. It is crucial to determine whether this fungus has the potential to cross-infect oil palm or just as a non-virulent rhizopheric fungal strain. The only negative aspect associated with *Fusarium* in oil palm was reported in relation to *Fusarium sacchari*, which is a moderately virulent strain but less prevalent in distribution, including Indonesia. This species is recognised as one of the causal agents responsible for crown spear rot (CSR) or crown disease in young oil palms (Suwandi *et al.*, 2012). On the contrary, it is also widely recognised that specific non-pathogenic strains of *Fusarium* can provide crop protection against *F. oxysporum* pathogens (Iida *et al.*, 2022). Thus, it is plausible to consider that a similar phenomenon may occur with *G. boninense*. Among the extensively studied cases, Fo47 (*Fusarium oxysporum*) stood out, as it established colonisation on the root surface and the surrounding soils in the vicinity of root epidermal cells. This colonisation enables Fo47 to effectively compete with pathogens for essential nutrients (Bolwerk *et al*., 2005).

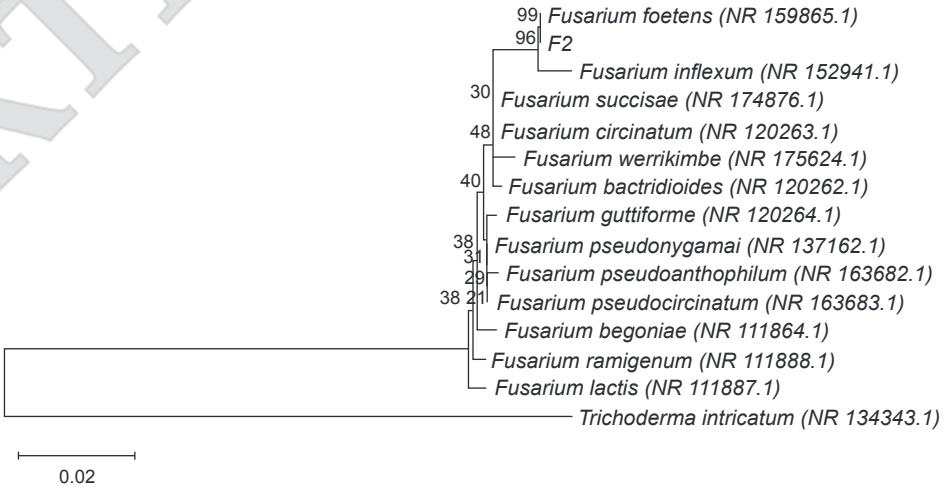

Figure 1. Phylogenetic tree of isolate F2 based on the alignment of ITS-rDNA from Fusarium *spp. and* Trichoderma intricatum *as an outgroup. Sequence alignment and tree construction were performed by MEGA11 using the neighbour-joining method and tested with 1000× bootstrapping.*

Through the disc vapour assay, the growth of the *G. boninense* colony was inhibited (*Figure 2*). The contactless exposure of *F. foetens* F2 to the pathogen demonstrated that inhibition occurred as a result of prolonged exposure to VOCs. The final observation on day 5 showed the highest growth inhibition of 63.64%, with the colony diameter reduced to 32 mm, which was much smaller compared to the control plate of *G. boninense* without VOCs (88 mm). However, it should be noted that studies examining *Fusarium* strains against *G. boninense* are still limited, as is the use of volatilisation assays to explore the potential of volatiles. Several studies have reported inhibition using VOCs from other microbial sources, particularly actinobacteria such as *Nocardiopsis alba* and *Streptomyces* spp., which exhibited varying levels of effectiveness (Budi *et al.*, 2022; Islamiati *et al.*, 2022; Widada *et al.*, 2021). The percentage of inhibition achieved through VOCs produced by a collection of peatland actinobacteria ranged between 4.47% and 20.23% against *G. boninense*, which was lower compared to this study (Budi *et al.*, 2022). According to Islamiati *et al.* (2022), an inhibition of 55% was achieved using the double-dish sets (DDS) method by exposing the colony of *Streptomyces* sp. GMR22 to the *G. boninense* colony. In a study conducted by Widada *et al.* (2022), the two-petri-dish method was employed, where *G. boninense* and *Nocardiopsis alba* strains were inoculated side by side. The highest inhibition rate, reaching 62.60%, was observed on the fifth day, which was similar to recent findings. Despite employing different inoculation methods, it can be inferred that the VOCs generated by antagonistic biocontrol agents possess considerable potential against *G. boninense*.

The ultrastructure of *G. boninense* was examined using SEM imagery after exposure to VOCs from *F. foetens* F2, and comparisons were made with the control (*Figure 3*). The treated mycelial network appeared thinner, wrinkled, but more compact compared to the control. This was evident from the hyphal arrangement, which showed reduced empty spaces. At 8000× magnification, the control mycelium exhibited a normal and intact mycelia, while the mycelia exposed to VOCs appeared shriveled and incomplete. This was observed through disrupted branching or potential degeneration within the mycelium. Fungal cell walls typically contain 50%-60% of the dry weight of the cell wall, with β-(1-3)-glucan being the predominant component (Latgé, 2007). This β-(1-3)-glucan constitutes up to around 40% of the total fungal cell volume and serves as a common target for antifungal compounds (Gow *et al.*, 2017). The antifungal activity of *F. foetens* VOCs is likely linked to the destabilisation of cell wall structures and cytoskeleton in *G. boninense*, particularly involving chitin, glucan, and tubulin proteins (Islamiati *et al.*, 2022; Takeshita *et al.*, 2014). The incomplete extension of hyphae in specific branches may be attributed to internal damage or interactions with the VOCs, the exact mechanisms of which are currently unknown. The combination of external and intracellular damage may lead to an imbalance in homeostasis, ultimately impeding the overall growth of the *G. boninense* colony.

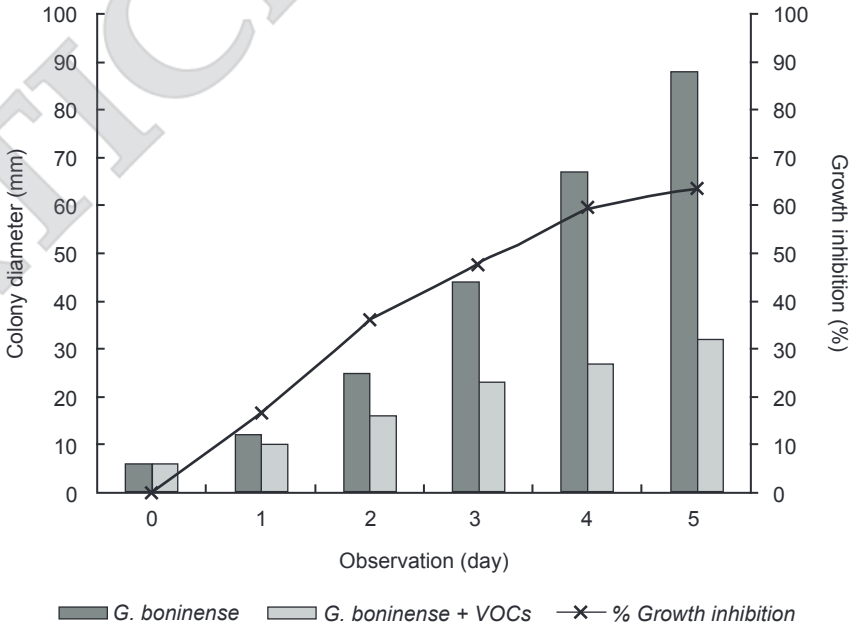

Figure 2. Inhibition of G. boninense *by volatile organic compounds (VOCs) of* F. foetens *F2.*

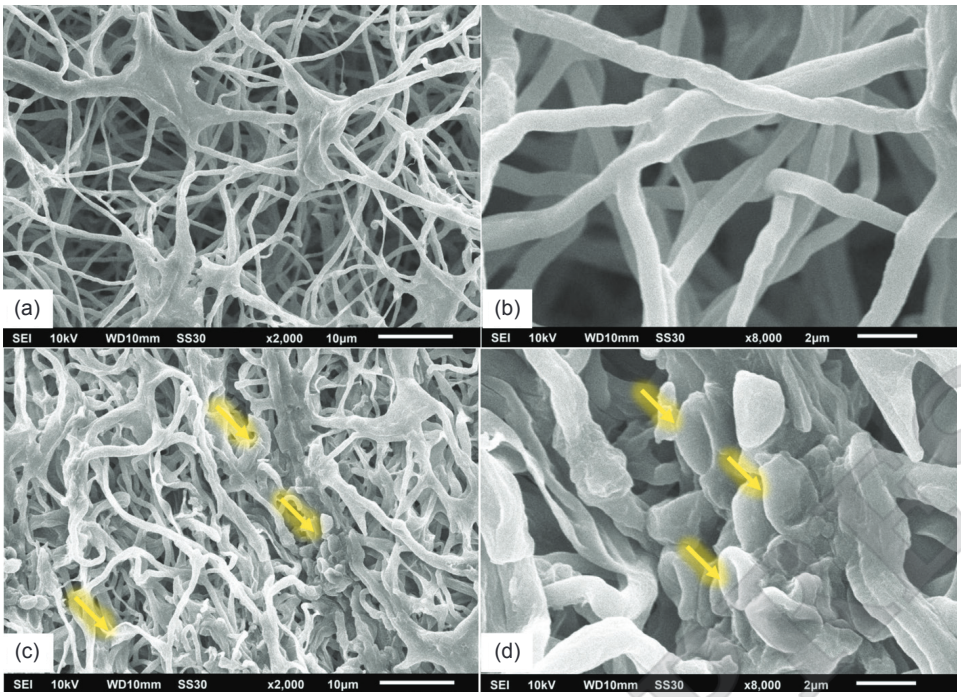

Figure 3. (a) The ultrastructure of G. boninense *in control plate at 2,000× and (b) 8,000× magnification. (c) The ultrastructure of* G. boninense *after 5-d exposure to VOCs of* F. foetens *F2 at 2,000× and (d) 8,000× magnification. The yellow arrows indicate visual evidence of hyphal abnormalities following VOC exposure, including shrinkage, contraction, and branch deformation.*

A total of 45 VOCs were identified and confirmed by comparing their similarity percentages with the database (*Figure 4*). Among these, four major components were identified based on their relative peak areas (%): 2,3-Pyrazinedicarboximide (15.54%), 2-(2-Butoxyethoxy) ethyl thiocyanate (11.43%), 3,5-Dimethoxyphenol (11.40%), Indolylmethylthiohydroximate (5.56%), and other minor compounds (<5.00%).

These compounds will be further analysed using an *in silico* approach as anti *G. boninense.* The compound, 2,3-Pyrazinedicarboximide or 5h-Pyrrolo[3,4-b]pyrazine-5,7(6h)-dione ($C_6H_3N_3O_2$) belongs to a class of heterocylic compounds or cyclic imides, which are valuable bioactive compounds with a wide range of biological activities (Hassanzadeh and Jafari, 2018). The hydrophobic nature of cyclic imides and their N-derivatives allows for enhanced membrane permeability, facilitating their interaction with intracellular targets. This properties is advantageous for their potential as drug candidates, as it increase their ability to reach their target sites within cells (Patil and Rajput, 2014). Specifically, 2,3-Pyrazinedicarboximide has not been reported or directly tested for its activity against *G. boninense* or its antifungal activity against other species. However, several studies have documented inhibitory activity of other derivatives and related cyclic imides against *Candida albicans*, *Candida krusei, Candida tropicalis, Geotrichum candidum, Trichophyton mentagrophytes*, and *Trichophyton rubrum* (Gayoso *et al.*, 2006; Sortino *et al.*, 2008). 2-(2-Butoxyethoxy) ethyl thiocyanate or lethane ($C_9H_{17}NO_2S$) is an organosulfur compound or isothiocyanate that is commonly used as insecticides (Tsao *et al.*, 2002). Isothiocyanates, in their various forms, have been recognised for their antifungal properties against a wide array of plant pathogens, particularly *Alternaria brassicicola, Fusarium oxysporum, Rhizoctonia solani*, and *Sclerotinia sclerotiorum*. Additionally, human-pathogenic strains such as those belonging to the *Candida* genus are also susceptible to these compounds. The mechanisms of action include growth inhibition, suppression of sclerotia or spore germination, and prevention of biofilm formation (Plaszko *et al.*, 2021). Therefore, based on the findings of this study, the compound produced by *F. foetens* could be regarded as the first report in VOCs. The phenol derivative, 3,5-Dimethoxyphenol ($C_8H_{10}O_3$) is characterised by the presence of two methoxy ($-OCH_3$) groups attached to the benzene ring at positions 3 and 5 that could be present as antifungal compound in VOCs. Phenols and phenolics are essential compound classes, recognised for their natural antifungal properties against *G. boninense*. Their mechanism of action involves the suppression of enzymes responsible for the degradation of wood and cell walls (Surendran *et al.*, 2017). In a more recent study by Fernanda *et al.* (2021), the inhibitory effects of benzoic acid, a phenol compound, was investigated at concentrations of 5 mM and above. The findings revealed that these concentrations

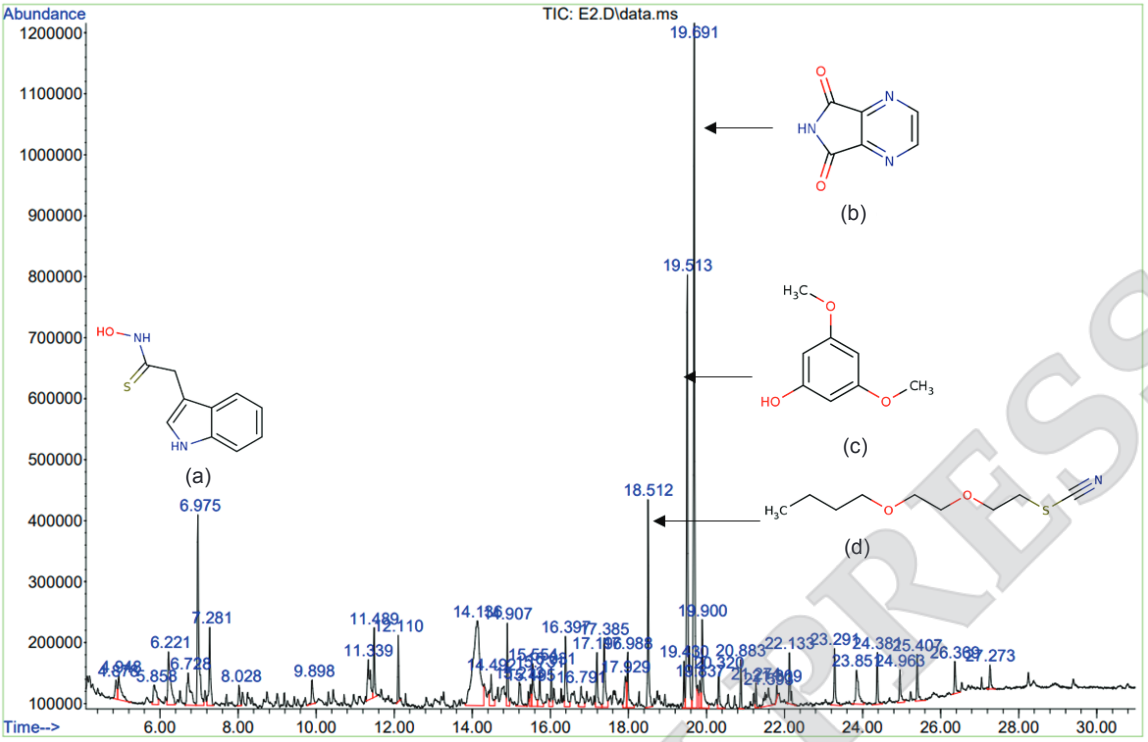

Figure 4. GC-MS spectra of volatiles emitted from F. foetens *F2. Chemical structure of four selected VOCs: (a) Indolylmethylthiohydroximate, (b) 2,3-Pyrazinedicarboximide, (c) 3,5-Dimethoxyphenol, and (d) 2-(2-Butoxyethoxy) ethyl thiocyanate. The numbers above the spectrum indicate the retention time (in min) of each identified compound.*

effectively hindered or delayed the establishment of the pathogen in oil palm wood, through cell mortality. Indolylmethylthiohydroximate or indolylmethylthiohydroximic acid ($C_{10}H_{10}N_2OS$) is a distinct form of glucosinolates that possesses an indole moiety (referred to as indole GSs), and it is currently known to exist as a component in the plant defence and abiotic stress response of *Arabidopsis thaliana* (Brader *et al.*, 2001; Zhao *et al.*, 2017). Some glucosinolates play a crucial role in defence against bacterial pathogens when activated upon pathogen attack (Rask *et al.*, 2000). However, there are currently no reports on the presence of indolylmethylthiohydroximate or its constituents as volatile organic compounds (VOCs) in fungi. It is then possible that the chemical cocktails found in the VOCs of *F. foetens* F2 may have the potential to be developed as antifungal agents in the future.

Xyloglucanases (XEG) are classified as cell wall-degrading enzymes (CWDEs) that specifically hydrolyse xyloglucan, a major component of hemicellulose in the plant cell wall, consisting of β-1,4-glucan linkages. This enzyme, categorised as a glycoside hydrolase, plays a significant role in phytopathogenic fungi, including possibly *G. boninense*. The enzyme can function as pathogen-associated molecular patterns (PAMPs), triggering PAMP-triggered immunity (PTI) (Zhang *et al.*, 2021). Interestingly, their PAMP activity may be separate from their enzymatic activity, as evidenced by *Verticillium dahlia* (Gui *et al.*, 2017). Through comprehensive genomic screening of various bacteria, fungi, oomycetes, and plant species, it has been observed that homologous protein sequences of XEG are exclusively present and widely distributed among microbial taxa, while absent in plants (Raflei *et al.*, 2021). Consequently, XEG becomes a potential target for antifungal treatment. As no native ligand was identified from the crystallography data, hexaconazole was then assigned as a standard antifungal agent in oil palm, as a reference, and compared it with four selected volatile organic compounds (VOCs) derived from *F. foetens*.

The interaction between hexaconazole and XEG protein resulted in a total of 16 bonds, including Van der Waals, π-alkyl, π-anion, and π-π stacked interactions. Van der Waals interactions were observed at three specific amino acid positions (Val-64, Ile-133, Phe-207) as depicted in *Figure 5*. No hydrogen bonds were formed during the simulation. Docking analysis of the ligand and XEG protein demonstrated a high-affinity value, indicated by an increased negative docking score (Du *et al.*, 2016). Hexaconazole exhibited a slightly lower affinity (-5.4 kcal/ mol) to the XEG protein compared to the other two VOCs, namely 2,3-Pyrazinedicarboximide (-5.5 kcal/ mol) and indolylmethylthiohydroximate (-5.5 kcal/ mol) (*Table 1*). Both VOCs showed comparable activity and affinity to the standard antifungal agent hexaconazole in the simulation. The interaction of

ligands with proteins through hydrogen bonds or Van der Waals interactions contributes to the stability of ligand-protein complexes (de Freitas and Schapira, 2017), which may lead to stronger antimicrobial activity. According to *Table 1*, all simulated VOCs from *F. foetens* exhibited a greater number of hydrogen bond interactions during protein-ligand interactions in comparison to hexaconazole. This indicates a propensity to function as more effective inhibitors of XEG activity due to their enhanced capacity for maintaining stability through hydrogen bonding interactions. The hydrogen bond interactions of 2,3-Pyrazinedicarboximide were observed with the amino acids Ser-36, Ser-56, and Ser-54, while indolylmethylthiohydroximate formed hydrogen bonds with Asp-26, Asp-105, and Glu-119 (*Figure 6*). Therefore, it is confirmed that 2,3-Pyrazinedicarboximide and Indolylmethylthiohydroximate may possess antifungal activities.

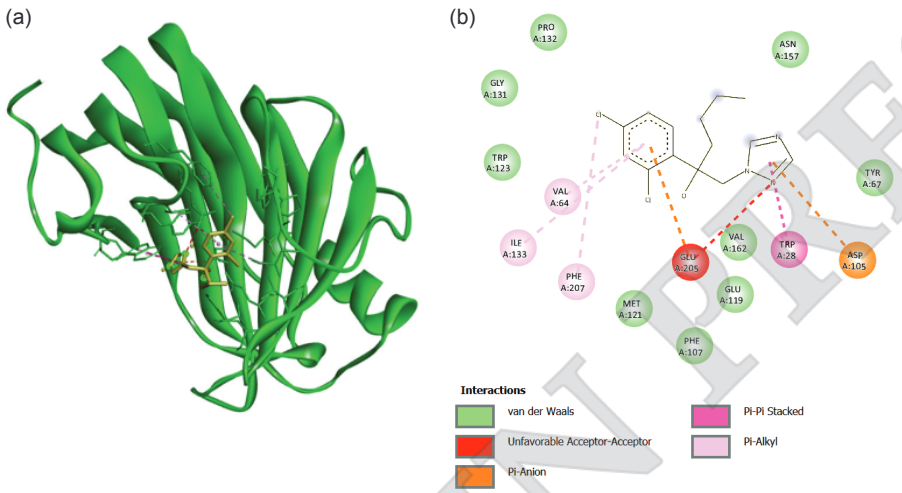

Figure 5. (a) Binding sites of the standard antifungi, hexaconazole (yellow) to xyloglucanaseprotein (green) and (b) Two dimensional (2D) visualisation of hexaconazole and xyloglucanaseprotein interaction.

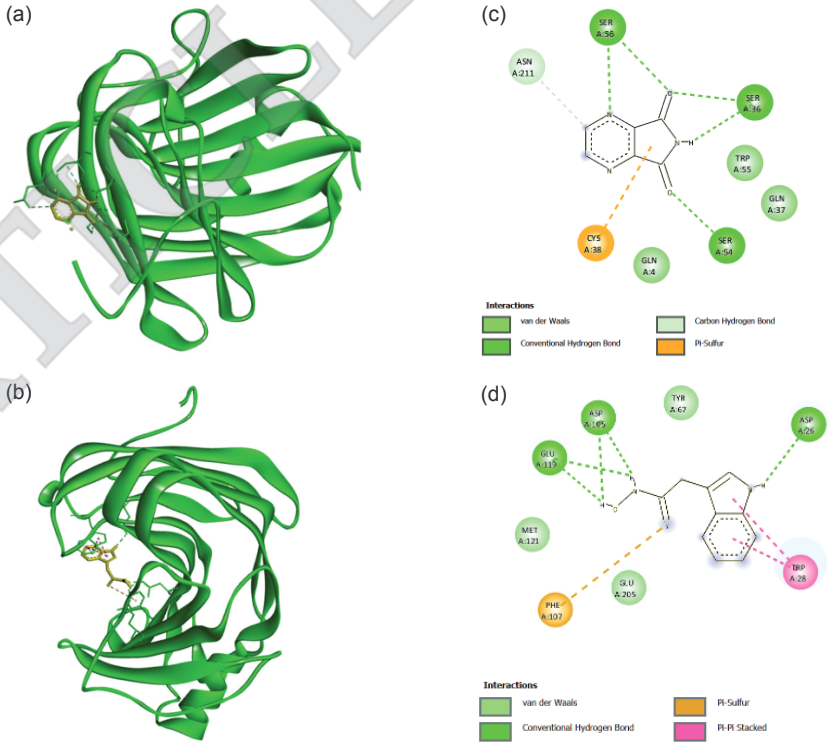

Figure 6. Docking visualisation of two volatiles by Fusarium foetens F2: (a) 2,3-pyrazinedicarboximide (yellow) and (b) indolylmethylthiohydroximate (yellow) to xyloglucanaseprotein (green). The 2D interaction of (c) 2,3-pyrazinedicarboximide and (d) indolylmethylthiohydroximate.

TABLE 1. BINDING ENERGY BETWEEN XEG PROTEIN AND HEXACONAZOLE AND SELECTED VOLATILE ORGANIC COMPOUNDS AS TEST OF LIGANDS

Compound	Molecular weight (g/mol)	No. of H bonds	Binding energy (kcal/mol)
Hexaconazole (Standard)	314.20	-	-5.4
2,3-Pyrazinedicarboximide	149.11	3	-5.5
Indolylmethylthiohydroximate	206.27	3	-5.5
3,5-Dimethoxyphenol	154.16	3	-4.7
2-(2-Butoxyethoxy)ethyl thiocyanate	203.30	2	-3.1

CONCLUSION

Fusarium foetens F2, a soil fungus found in healthy oil palm plantations, emit volatile organic compounds (VOCs) that exhibit potent antifungal activity against *G. boninense*. These VOCs effectively inhibit the growth of *G. boninense* and cause both external and internal damage to its cells. Among the VOCs emitted by the fungus, four major compounds have been identified as the most prevalent. Notably, 2,3-Pyrazinedicarboximide and indolylmethylthiohydroximate have displayed a strong binding affinity to XEG protein, a virulent factor of *G. boninense*, as revealed by molecular docking simulations. The results indicate a potential candidate for the future development of biocontrol and antifungal agents for managing BSR in oil palm.

ACKNOWLEDGEMENT

Financial support for the research was provided by LPDP - the Ministry of Finance of the Republic of Indonesia (RIIM - Technical Program No. PRN-018512262) for 2022-2024 years. The research was also supported by the facilities, scientific and technical support from the Laboratory of Biotechnology, National Research and Innovation Agency through *E-Layanan Sains*-BRIN.

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