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ANTIFUNGAL ACTIVITY OF ETHANOLIC EXTRACT FROM INDIGENOUS BACTERIUM *Bacillus subtilis* STRAIN MN704394.1 CULTURE CONTAINING EICOSANE AND ETHYL STEARATE AGAINST *Ganoderma boninense*

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ABSTRACT

Ganoderma boninense *is a fungal pathogen responsible for causing basal stem rot disease in oil palm. This study focuses on exploring the biocontrol potential of indigenous bacterium by detecting several bioactive compounds capable of inhibiting the growth of* G. boninense. *The 16S rRNA sequence analysis proved that the bacterium was* Bacillus subtilis *strain MN704394.1. The ethanolic extracts of bacterium culture supernatant, spanning 8 to 24 hr, were collected and tested on their inhibitory effects against* G. boninense *using simultaneous, preventive, and curative methods. The one obtained at the 8th hour using the preventive method demonstrated the highest inhibition percentage at 96.44%. By using GC-MS analysis, three compounds were consistently detected at all time points, which are eicosane, ethyl stearate and methyl palmitate. Eicosane and ethyl stearate were identified as bioactive compounds. Specifically, at the 8th hr, eicosane constituted 68.7467% of the total area, while ethyl stearate accounted for 10.3018%. Eicosane, a straight-chain alkane consisting of 20 carbon atoms, exhibited antifungal activity. Ethyl stearate, belonging to the group of fatty acid esters, demonstrates significant inhibitory activity against* G. boninense. *The potential of these two compounds is substantial, with prospects for further development, particularly in controlling* G. boninense.

Keywords: *Bacillus subtilis*, eicosane, ethyl stearate, *Ganoderma boninense*, oil palm.

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INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is a vital commodity for Indonesia's economy due to its substantial contribution as a primary source of vegetable oil (Latifah & Kadir, 2021). However, the prevalence of basal stem rot disease caused by *Ganoderma boninense* presents a significant challenge to Southeast Asia's oil palm cultivation, accounting for 86% of global palm oil production, particularly in Indonesia (Wong et al., 2022). The disease leads to reduced productivity, with affected trees experiencing a decline in both quantity and quality of fruit bunches, subsequently diminishing oil yield (Evizal & Prasmatiwi, 2022). In certain regions of Sumatra Island, Indonesia, disease incidence rates have been reported spanning from 37% to 52%, intensifying concerns about production losses (Paterson, 2019). Notably, *G. boninense*-infected oil palm trees yield as low as 4 t of crude palm oil ha^{-1} yr^{-1}, considerably lower than the potential 19 t ha^{-1} yr^{-1} under healthy conditions (Rebitanim et al., 2020).

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To address this challenge, biological control using environmentally friendly strategies, particularly through biofungicides, has gained prominence in the oil palm industry. *Bacillus subtilis* emerges as a compelling bioagent due to its effective antimicrobial activity against various pathogens in agricultural contexts (Chandrasekaran & Chun, 2016; Zhang et al., 2022). Its colonisation of plant roots inhibits *G. boninense* growth by producing antimicrobial substances and engaging in competitive interactions (Puspita et al., 2019).

Exploration of *B. subtilis* metabolites reveals the presence of eicosane compounds in strain BS-01 and fatty acid ester compounds in strain SVUNM4 (Awan et al., 2023; Sreenivasulu et al., 2017). While eicosane's potential as an antifungal against *G. boninense* remains understudied, its high flash point poses challenges for storage. Similarly, ethyl stearate, identified among the fatty acid esters, has exhibited inhibitory activity against *Candida albicans* (Huang et al., 2010). The main mechanism of fatty acids (ethyl stearate) as antifungals is through the insertion of fatty acids into the fungal lipid bilayer membrane which damages membrane integrity, causes uncontrolled release of electrolytes and intracellular proteins, and ultimately causes disintegration of the fungal cell cytoplasm. The mechanism of the compound as an antifungal can be that the compound spreads through the fungal membrane and interferes with the synthesis of important components such as ergosterol, glucan, chitin, protein and glucosamine (Tay & Chong, 2016). Interestingly, higher concentrations of these metabolites are found in oil palm plants displaying resistance to *G. boninense*, hinting at their potential as effective antimicrobial agents (Said et al., 2015).

This study explores the biocontrol potential of *B. subtilis* strain MN704394.1 against *G. boninense*, shedding light on bioactive compounds inhibiting its growth. The investigation is significant considering the adverse impact of *G. boninense* on oil palm productivity and introduces potential antifungal agents, aiming to address their limited research and storage challenges.

MATERIALS AND METHODS

Time and Location

The research was conducted from June 2022 to April 2023 at the Biotechnology and Chemistry Laboratory, National Research and Innovation Agency, South Tangerang City, Banten Province, Indonesia.

Biological Materials and Molecular Identification

The bacterium was isolated from healthy oil palm tissue in susceptible areas to serve as stock isolates. The bacterium's stock culture was routinely maintained in the Laboratory for Biotechnology, National Research and Innovation Agency, South Tangerang City, Banten Province, Indonesia. The *G. boninense* strain SSU008, a pathogenic fungus used in this study, was obtained from the collection at Indonesian Oil Palm Research Institute (IOPRI), Marihat, Simalungun Regency, North Sumatra Province, Indonesia. To rejuvenate the stock cultures of indigenous microbes, specific solid media were employed. Nutrient agar (NA) medium was utilised for the bacterium, while potato dextrose agar (PDA) medium was used for *G. boninense*. To identify the antagonistic bacterium strain, DNA extraction was performed using Instagene™ Matrix, followed by sample amplification using 16S rRNA primers (5'-AGA GTT TGA TCC TGG CTC AG-3') and (5'-GGA TAC CTT GTT ACG ACT T-3') (Ibrahim et al., 2016) with a base length of 1,500 base pairs. The amplified sample was then sequenced by 1st Base and further analysed with ClustalW and MEGA6 programme to construct a phylogenetic tree (Tamura et al., 2013).

Bacterial Growth Curve

Bacterial growth measurement commenced with a pre-culture step, involving the inoculation of 1-2 colonies of *B. subtilis* MN704394.1 into 50 mL of nutrient broth (NB) media. From the pre-culture, 1 mL was reinoculated into 150 mL of NB media and incubated on a shaker at 150 rpm and 27°C. The total plate count (TPC) method was employed to measure bacterial growth, with measurements taken every 4 hr over a 28 hr period.

To perform the measurement, 1 mL of the bacterial culture was mixed with 9 mL of a 0.85% NaCl physiological saline solution in a test tube. Serial dilutions were prepared from 10^{-1} to 10^{-8} in duplicates. Duplicates from the 10^{-5} to 10^{-8} dilutions, at 0.1 mL each were spread onto nutrient agar (NA) plates. The plates were incubated at 27°C for 24 hr (Irma et al., 2018).

Subsequently, microbial colonies grown on each sample plate were counted using a colony counter. Data analysis involved describing the TPC results for each sample, which were presented using the standards plate counts (SPC) in a table for clarity. SPC is a method used to determine the microbial count within the range of 30–300 colony forming units (CFUs) from dilutions 10^{-5} to 10^{-8}. This approach was employed to minimise potential errors in the analysis process, particularly statistical errors (Yunita et al., 2015).

Extraction of Bioactive Compounds

Bacillus subtilis strain MN704394.1 was cultivated in NB medium. One or two colonies of the bacteria were inoculated into 100 mL of NB medium to create a starter culture. Subsequently, 10 mL was further inoculated into 2 L of NB medium. The bacterial culture was harvested at specific time points, during both the exponential and stationary growth phases, totalling 100 mL of culture. Bacterial cells were separated from the supernatant by centrifugation at 10 000 rpm for 10 min. Furthermore, 100 mL of the supernatant was mixed with 100 mL of ethanol (1:1 v/v) as solvent. To extract metabolites with a reasonably wide polarity spectrum, ethanol solvent is used, as presented by Chan and Chong (2020) and Liu et al. (2007). The resulting mixture was added to a separating funnel and shaken for 15 min, forming two distinct layers. The upper layer consisted of the organic solvent, while the lower layer contained the medium and cell biomass (pellet). The organic solvent layer was concentrated using a rotary vacuum evaporator at approximately 40°C. The obtained extract was then dried at room temperature to remove any remaining solvent (eluent) (Irma et al., 2018).

Activity Test of Crude Extracts Against *G. boninense*

The activity testing of the bacterial extract aimed to identify an extract that effectively inhibited the pathogenic fungus. This involved employing the agar well diffusion method by creating wells on PDA media using a cork borer. The tests were conducted simultaneously, both preventively (pre-infection) and curatively (post-infection) (Syed-Ab-Rahman et al., 2019).

Prior to subjecting the bacterial extract to *G. boninense*, it was dissolved in methanol at a concentration of 10,000 ppm (10 mg 100 mL^{-1}). The supernatant extract from the bacterial culture was introduced into the created wells. Subsequently, *G. boninense* fungus, measuring approximately 1 cm in size, was placed on the PDA media adjacent to the well containing the extract. For comparative purposes in the extract testing, both positive and negative controls were utilised. Benzoic acid (Merck), at the same concentration as the extract, served as the positive control and was dissolved in methanol at a concentration of 10,000 ppm (10 mg 100 mL^{-1}). The negative control involved the application of methanol without the addition of the extract. The inhibition percentage (IP) of the bacterial extract was quantified following the method described by Irma et al. (2018) using the formula: $IP = (R_1 - R_2)/R_1 \times 100\%$, where R_1 signifies pathogen growth in the control and R_2 indicates pathogen growth in the dual culture treatment.

Profiling Bioactive Compounds Using GC-MS

The ethanol extract of *B. subtilis* strain MN704394.1 underwent compound identification through GC-MS analysis at the Chemistry Laboratory, National Research and Innovation Agency, South Tangerang City, Banten Province. GC-MS analysis of the bacterial extract was performed using an Agilent 7890 B gas chromatograph coupled in tandem with MSD 5977 A mass spectrometer. A total of 1 µL volume of the extract solution was injected into a capillary column measuring 30 m × 250 µm × 0.25 µm (Agilent, Type 19091S-433: 93.92873 DB-5MS UI, 5% phenyl methyl silox). The initial oven temperature was set to 40°C with a 1 min hold time, then gradually ramped up at a rate of 10°C min^{-1} until reaching 300°C, where it was maintained for 4 min. Helium served as the carrier gas at a flow rate of 1 mL min^{-1}. The injection mode used was splitless, with an injector temperature of 250°C. The detector employed was a mass-spectrometry (MS) system with ion source and interface temperatures set at 230°C and 250°C, respectively. For the analysis process, electron impact (EI) ionisation was utilised with an ionisation energy of 70 eV. Data processing was performed using the GC-MS Postrun Analysis software. The peak constituents were cross-referenced with the data available in the NIST-17 (National Institute of Standards and Technology) mass spectral library.

RESULTS AND DISCUSSION

Analysis via BLAST of all consensus sequences revealed a high degree of similarity and substantial query coverage between the bacterial strain and the 16S rRNA sequence in the NCBI database. Numerous similar sequences were retrieved from the BLAST analysis and employed for both the reconstruction of a phylogenetic tree and the analysis of genetic distances. Phylogenetic analysis using the neighbour-joining method conclusively identified the bacterial isolate as *B. subtilis* strain MN704394.1, demonstrating 100% sequence similarity (*Figure 1*). According to established taxonomic criteria, species are delineated when the similarity exceeds 97%, species identification is considered a "match" when the similarity surpasses 99% (Drancourt et al., 2000; Janda & Abbott, 2007), while any similarity below 97% suggests the possibility of a novel species (Stackebrandt & Goebel, 1994).

Growth assessment of *B. subtilis* strain MN704394.1 was conducted at 4 hr intervals during a 24 hr period, utilising the TPC calculation method and NB media for bacterial incubation. *Figure 2* shows the growth curve indicating that *B. subtilis*

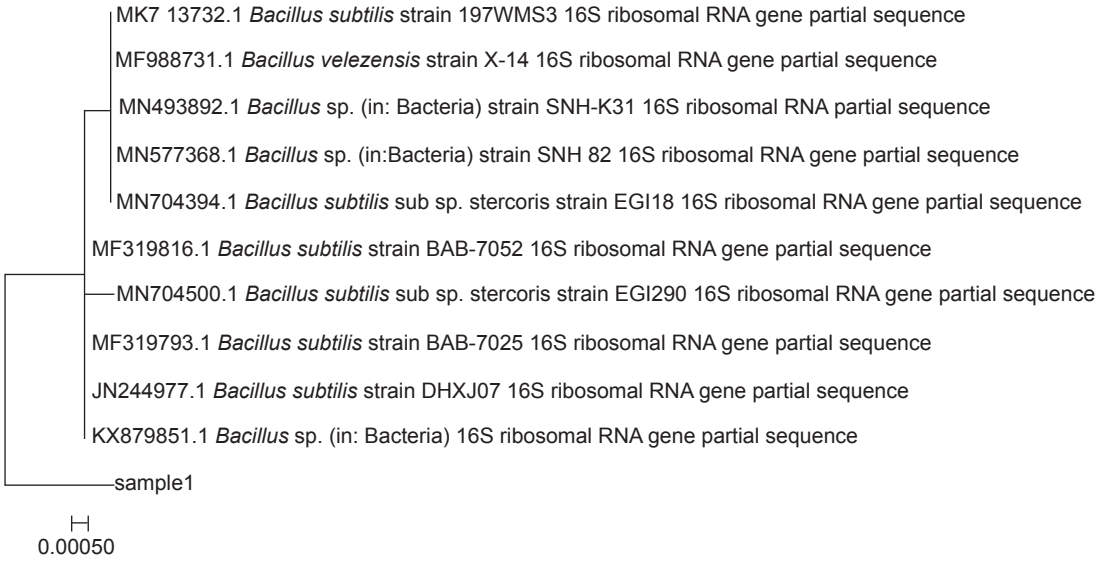


Figure 1. Phylogenetic tree depicting the bacterial isolate based on the 16S rRNA gene.

strain MN704394.1 displayed a brief lag phase, facilitating its rapid entry into the logarithmic (exponential) phase, which was notably extended. This pattern was discernible from hr 0 to hr 12, during which bacterial cell counts increased from 6.550×10^7 to 2.595×10^8 CFU mL^{-1}. Following the attainment of the logarithmic phase, a stationary phase ensued and persisted until the 20th hr, with bacterial cell counts at the 20th hr (2.515×10^8 CFU mL^{-1}) showing no significant deviation from those at the 12th and 16th hr. Subsequently, a death phase commenced, continuing until the 28th hr, characterised by a decline in bacterial cell numbers. *Bacillus subtilis* strain MN704394.1 exhibited decreasing cell counts until the 28th hr, reaching 1.205×10^8 CFU mL^{-1}. These bacterial growth phases served as reference points for determining the optimal time for bacterial cell harvest in the extraction process. In this treatment, bacterial cell harvesting was performed between the 8th and 24th hr.

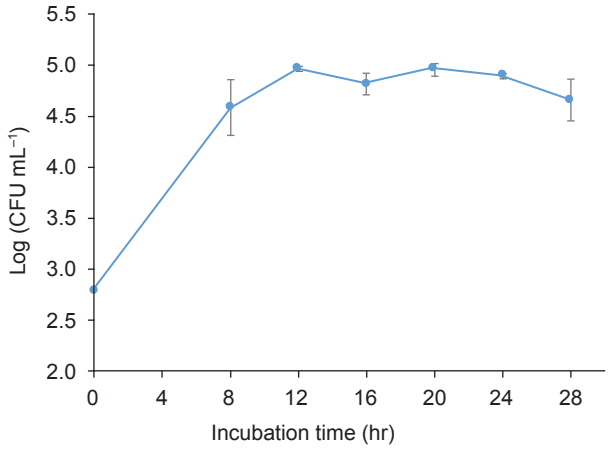


Figure 2. Growth curve of B. subtilis *strain MN704394.1 on NB media (Each data point represents the average of two values and error bars depict the standard deviation).*

The measurement of growth curves aimed to ascertain the optimal time for metabolite production during bacterial proliferation. The bacterial growth curve elucidates the distinct phases in bacterial proliferation, encompassing the lag phase (adaptation phase), the logarithmic phase (exponential phase), the stationary phase and the decline phase (death phase) (Wang et al., 2015). The growth curve analysis revealed that the *B. subtilis* strain MN704394.1 exhibited a brief lag phase. This phenomenon arose due to the pre-culture preparation conducted before growth curve assessment, expediting bacterial growth and propelling it into the logarithmic phase.

The lag phase or adaptation phase in bacterial growth is influenced by new environmental conditions and the initial inoculum size (Schultz & Kishony, 2013). When testing the bacterial growth curve under the same growth environment, the bacteria do not require much time to adapt to their surroundings, thus accelerating their entry into the logarithmic phase (Irma et al., 2018). The logarithmic phase in *Figure 2* observed in *B. subtilis* strain MN704394.1 indicates an increase in the number of bacterial cells until the 12th hr. According to Sulistiana et al. (2021), the mass and volume of cells increased during the logarithmic phase. Andriani et al. (2017) also stated that during the logarithmic phase, there is regular cell division where bacteria double their cell numbers at a constant rate, maintain constant metabolic activity and experience balanced growth conditions.

The logarithmic growth phase yields beneficial metabolites crucial for bacterial growth. Primary metabolites, the products of metabolic pathways, play a pivotal role in microbial growth. Ethyl stearate qualifies as a primary metabolite as it emerges during the logarithmic phase of *B. subtilis* strain MN704394.1, rendering it

indispensable for this bacterium's growth. The ethyl stearate quantity increases throughout the logarithmic phase (0th to 12th hr) and stabilises during the stationary phase (12th to 20th hr). This observation aligns with Rezvani et al. (2017) findings, which highlighted the production of essential primary metabolites by *Lactobacillus* sp. during the logarithmic phase (6th to 30th hr).

According to Azizah et al. (2015), *B. amyloliquefaciens* SAHA 12.07 underwent the logarithmic phase until the 6th hr, during which it produced enzymes capable of lysing fungal cell walls. *Bacillus subtilis* entered the stationary phase from the 12th hr until the 20th hr, followed by the death phase. During the stationary phase, the number of dividing cells equalled those succumbing to nutrient depletion (Wang et al., 2015). Secondary metabolites, such as antibiotics or other bioactive compounds toxic to microorganisms, were produced during this phase and acted as a defence mechanism against unfavourable conditions (Irma et al., 2018).

Li et al. (2016) reported that *B. amyloliquefaciens* produced antifungal metabolites during both the logarithmic and stationary phases. These metabolites served as a defence mechanism against extreme environmental conditions, leading to the subsequent death phase. During this phase, there was a decline in cell numbers due to the accumulation of excess toxins and nutrient depletion, resulting in a higher proportion of bacterial cells dying (Irma et al., 2018). Wang et al. (2015) also noted that during the death phase, bacteria lost their ability to divide, with the number of dead cells surpassing the live ones.

The crude extract was derived from bacterial culture supernatant via ethanol extraction, encompassing phases from logarithmic growth to the death phase. Ethanol extracts of *B. subtilis* strain MN704394.1, obtained at 8th, 12th, 16th, 20th and 24th hr, and dissolved in methanol at a concentration of 10,000 ppm, were assessed for their efficacy against *G. boninense*.

Results revealed that the crude extract from bacterial culture spanning 8 to 24 hr, utilising simultaneous, preventive, and curative methods, displayed inhibitory effects on *G. boninense*. Among these approaches, the simultaneous method exhibited the highest inhibition of *G. boninense* growth at the 8th hr in comparison to other time points (*Figure 3*). The inhibition percentage of the ethanol extract at the 8th hr in the simultaneous method was 79.47% (*Table 1*). In the preventive method, the most pronounced inhibition of *G. boninense* growth was also observed at the 8th hr, with an inhibition percentage of 96.44% (*Table 1*). This 8th hr inhibition in the preventive method represented the highest inhibition value among the three methods (*Figure 4*). Meanwhile, in the curative method, the highest inhibition of *G. boninense* growth was noted at the 20th hr when compared to other time points (*Figure 5*). The inhibition percentage of the ethanol extract at the 20th hr in the curative method was 50.76% (*Table 1*). Positive and negative controls were employed as reference points during the extract evaluation. The positive control, utilising benzoic acid alone, demonstrated inhibition solely in the curative method, with an inhibition percentage of 9.85%, whereas the negative control, employing methanol, exhibited no inhibitory activity.

To assess the influence of *B. subtilis* strain MN704394.1 bacterial culture extract on *G. boninense*, three methods were employed: Preventive, curative, and simultaneous (Syed-Ab-Rahman et al., 2019). The preventive method aims to avert fungal infections in plants by applying the bacterial culture extract before planting *G. boninense*. Conversely, the curative method provides substances or compounds at the site of fungal infection after planting *G. boninense* to halt its further development. The simultaneous method involves applying substances or compounds simultaneously with the planting of the pathogenic fungus (Doble & Kumar, 2005).

TABLE 1. INHIBITION PERCENTAGE OF ETHANOL EXTRACT FROM *B. subtilis* STRAIN MN704394.1 CULTURE AGAINST *G. boninense* GROWTH

No.	Extract	Inhibition percentage (%)		
		Simultaneous	Preventive	Curative
1	Negative control (without extract)	0.00 ± 0.00	0.00 ± 0.00	9.85 ± 1.07
2	Positive control (benzoic acid)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
3	Extract at 8 hr after incubation	79.47 ± 3.54	96.44 ± 5.04	50.00 ± 0.00
4	Extract at 12 hr after incubation	68.94 ± 2.79	94.02 ± 1.61	48.48 ± 4.29
5	Extract at 16 hr after incubation	74.24 ± 1.29	91.52 ± 8.14	44.70 ± 5.36
6	Extract at 20 hr after incubation	73.26 ± 4.61	90.45 ± 2.36	50.76 ± 5.36
7	Extract at 24 hr after incubation	70.83 ± 5.89	87.80 ± 6.54	49.24 ± 7.50

Note: ( ) - no inhibition observed; values are presented as the average of duplicate data standard ± deviation.

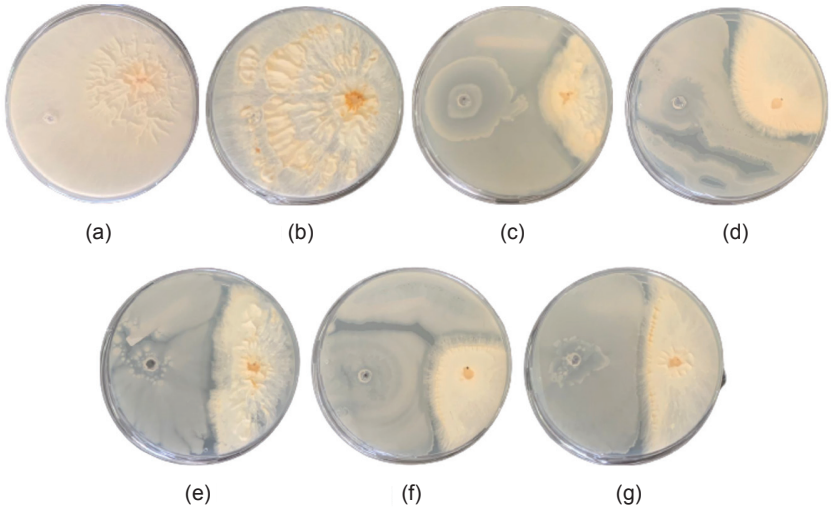


Figure 3. Antifungal activity of ethanol extract on simultaneous growth of G. boninense. *(a) Negative control, (b) positive control, ethanol extract at (c) 8th, (d) 12th, (e) 16th, (f) 20th and (g) 24th hr.*

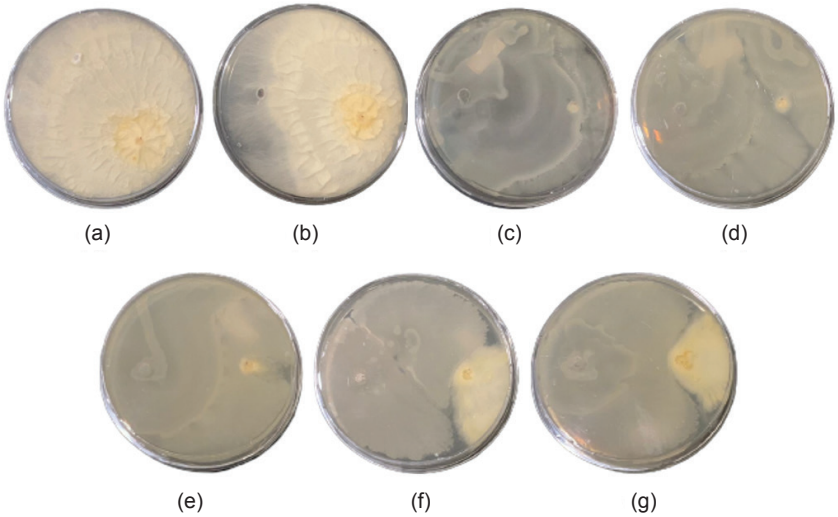


Figure 4. Antifungal activity of ethanol extract on preventive growth of G. boninense. *(a) Negative control, (b) positive control, ethanol extract at (c) 8th, (d) 12th, (e) 16th, (f) 20th and (g) 24th hr.*

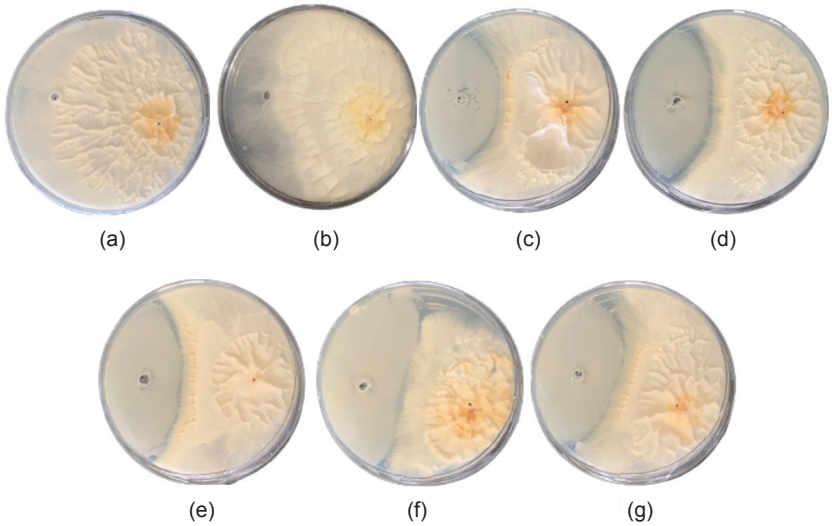


Figure 5. Antifungal activity of ethanol extract on curative growth of G. boninense. *(a) negative control, (b) positive control, ethanol extract at (c) 8th, (d) 12th, (e) 16th, (f) 20th and (g) 24th hr.*

Ethanol serves as the solvent for extracting the *B. subtilis* strain MN704394.1 culture. Chen et al. (2015) indicated that lower ethanol concentrations enhance the solubility of polar antifungal compounds, while higher ethanol concentrations were suitable for extracting semi-polar or non-polar compounds. This range of solvent polarities effectively extracted the antifungal compounds present in the bacterial culture supernatant. The concentrated extract resulting from this process was evaporated to prevent any potential interference during testing of the bacterial extract's activity. Additionally, methanol was employed in the extract testing due to its efficient dissolution of chemical compounds in the bacterial extract, ensuring complete dissolution of any residue adhering to the tube walls (Irma et al., 2018).

The extraction of supernatant from *B. subtilis* strain MN704394.1 cultures using the preventive method exhibited substantial inhibition, particularly at the 8th hr, achieving a remarkable 96.44% inhibition of *G. boninense*. Further application of the simultaneous method at the 8th hr resulted in 79.47% inhibition, followed by the curative method at the 20th hr, displaying a 50.76% inhibition. Bacterial extracts of pronounced efficacy yield more abundant metabolite outcomes, which can be efficiently extracted using ethanol, thus producing significant inhibitory effects.

The produced metabolites exhibit antifungal properties, evident from their inhibitory activity against fungal growth during extraction testing. Factors affecting bacterial growth include the nutrients in the media and environmental conditions, such as temperature, agitation, and pH (Irma et al., 2018). These factors influence bacterial metabolism in synthesising specific metabolite products. The culture supernatant extract of *B. subtilis* strain MN704394.1 at 8th hr was obtained from bacterial cultures in the logarithmic growth phase. Generally, primary metabolites such as enzymes and fatty acids can be produced during this phase (Awan et al., 2023; Azizah et al., 2015). However, secondary metabolite products, usually formed in the stationary phase, can also be produced in the middle of the logarithmic phase (Zvanych et al., 2014). The coarseness of the supernatant from *B. subtilis* strain MN704394.1 culture imparts antifungal properties against *G. boninense* growth by disrupting cellular activities, leading to inhibited fungal growth. The metabolite compounds produced by the bacteria elicit inhibitory responses on the growth of the pathogenic fungus (Irma et al., 2018). These findings demonstrated the effectiveness of compounds generated by *B. subtilis* in hindering *G. boninense* growth. As a positive control, benzoic acid was employed. Benzoic acid, widely used for its antifungal properties and preservation capabilities in various human consumption products, was chosen as a positive control (Loya-Rodriguez et al., 2023). In contrast, the negative control using methanol yielded different results from both the positive control and the treatments, as it did not exhibit any toxic effects on *G. boninense* mycelium growth.

The ethanol extract from *B. subtilis* strain MN704394.1 underwent GC-MS analysis to identify its bioactive compounds. Three compounds were consistently detected at all time points (8th, 12th, 16th, 20th and 24th hr): Eicosane, ethyl stearate, and methyl palmitate. Eicosane and ethyl stearate were identified as the predominant bioactive compounds across all time points and were present at elevated concentrations. Specifically, at the 8th hr, eicosane constituted 68.75% of the total area (at a retention time of 20.32), while ethyl stearate accounted for 10.30% (at a retention time of 22.12). The GC-MS analysis of the ethanol extract from *B. subtilis* strain MN704394.1 at 8th, 12th, 16th, 20th and 24th hr consistently revealed that ethyl stearate had the second-largest area value at each time point. At the 8th hr, ethyl stearate was identified with an area value of 10.30% at a retention time of 22.12 (*Table 2*). At the 12th hr, in the bacterial ethanol extract, ethyl stearate was found to have an area value of 9.02% at a retention time of 22.12 (*Figure 6*). Meanwhile, the GC-MS analysis of the ethanol extract from *B. subtilis* strain MN704394.1 at the 16th, 20th and 24th hr consistently showed that ethyl stearate maintained the same area value of 11.11% at a retention time of 22.12 (*Figure 6*).

Analysis of the crude extract from *B. subtilis* strain MN704394.1 using GC-MS revealed the consistent presence of three compounds across all monitored time points (8th, 12th, 16th, 20th and 24th hr). These compounds are eicosane, ethyl stearate, and methyl palmitate.

Prior investigations have substantiated the bioactivity of these aforementioned compounds. In a study conducted by Awan et al. (2023), compounds extracted from *B. subtilis* BS-01 and identified through GC-MS analysis yielded eicosane, palmitic acid and stearic acid compounds, displaying substantial potential as antifungal agents against *Alternaria solani*. Most fatty acids, including fatty acid ethyl ester (ethyl stearate) and fatty acid methyl ester (methyl palmitate), exhibit antifungal properties (Astiti & Ramona, 2021).

According to Tay and Chong et al. (2016), fatty acid esters identified via GC-MS analysis in papaya leaf extracts demonstrate potential inhibitory activity against *G. boninense*. One such compound within this group is ethyl stearate, an ethyl ester derivative with a 20-carbon chain. Additionally, fatty acid ethyl esters have exhibited inhibitory and antifungal properties against *Candida albicans*

TABLE 2. THE PROFILING OF BIOACTIVE COMPOUNDS IN THE ETHANOL EXTRACT AT THE 8TH HR USING GC-MS

No.	Retention time	Area (%)	Compounds	Formula	MW (g/mol)	Bioactivity
1	6.27	1.77	Cyclopentane, 1,2,3,4,5-pentamethyl-	$C_{10}H_{18}$	138.3	-
2	17.18	0.54	Tetracosane, 1-iodo-	$C_{24}H_{49}I$	464.6	-
3	17.90	6.65	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	$C_7H_4N_2O_2$	154.2	Antifungal (Sanjenbam & Krishnan, 2016), antibacterial, antioxidant (Kiran et al., 2018)
4	18.29	0.77	2-Propenamide	C_3H_5NO	71.1	-
5	19.43	0.46	(2-Piperidin-3-ylethyl)amine	$C_7H_{16}N_2$	128.2	Antifungal (Kunzler et al., 2013)
6	19.58	0.82	Palmitic acid, methyl ester	$C_{17}H_{34}O_2$	270.5	Antifungal (Pinto et al., 2017), antibacterial (Shaaban et al., 2021)
7	20.04	2.07	Nonadecane, 3-methyl-	$C_{20}H_{42}$	282.5	Antibacterial (Kumari et al., 2019)
8	20.32	68.75	Eicosane	$C_{20}H_{42}$	282.5	Antifungal (Awan et al., 2023), antibacterial (Wijayanti and Dewi, 2022)
9	20.72	0.40	3-Amino-2-ethyl-butyric acid	$H_{13}NO_2C_6$	131.17	-
10	20.89	0.47	Sarcosine, n-hexanoyl-, hexadecyl ester	$C_{25}H_{49}NO_3$	411.7	-
11	21.27	0.39	Cyanoacetylurea	$C_4H_5N_3O_2$	127.1	-
12	22.12	10. 30	Stearic acid, ethyl ester	$C_{20}H_{40}O_2$	312.5	Antifungal (Tay & Chong, 2016), antioxidant. antiinflammation (Ganesh & Mohankumar, 2017)
13	23.94	0.57	Sarcosine, N-isobutyryl-, tetradecyl ester	$C_{21}H_{41}NO_3$	355.6	-
14	24.38	0.61	3,6-Bis-dimethylaminomethyl-2,7-dihydroxy-fluoren-9-one	$C_{19}H_{22}N_2O_3$	326.4	-

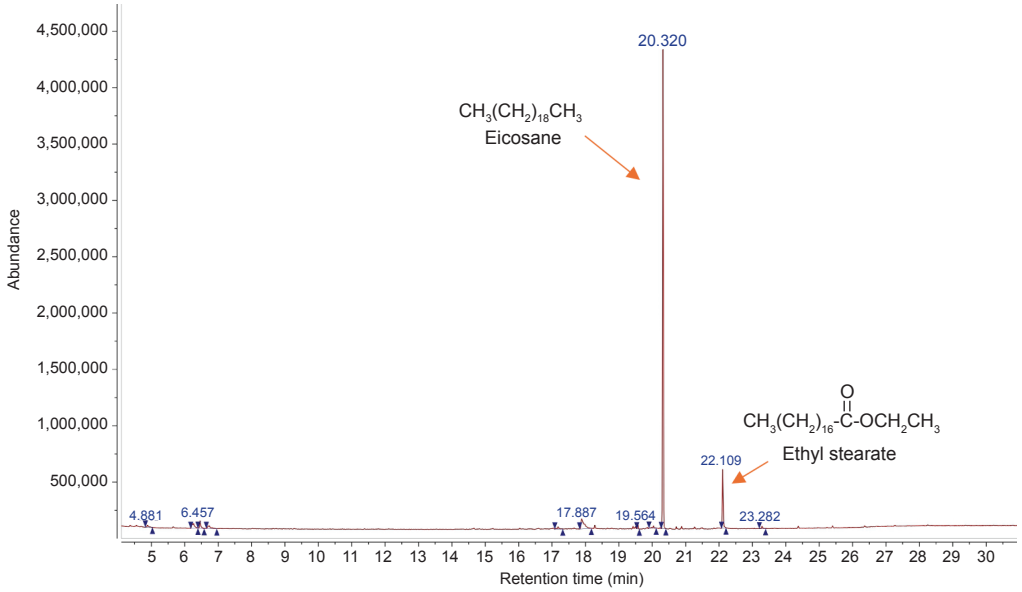
Note: MW - molecular weight.

(Huang et al., 2010). Pinto et al. (2017) have asserted that fatty acid esters, particularly against fungi, possess antimicrobial activity. These esters, identified through GC-MS analysis, were also found in the bacterium *B. subtilis* strain SVUNM4 (Sreenivasulu et al., 2017). Furthermore, Said et al. (2015) have reported that fatty acid esters, identified via GC-MS analysis, are most concentrated in healthy oil palm plants that are resistant to the pathogenic fungus *G. boninense*. These elevated levels of metabolite compounds in *G. boninense*-resistant oil palm plants may serve as potent antimicrobial agents.

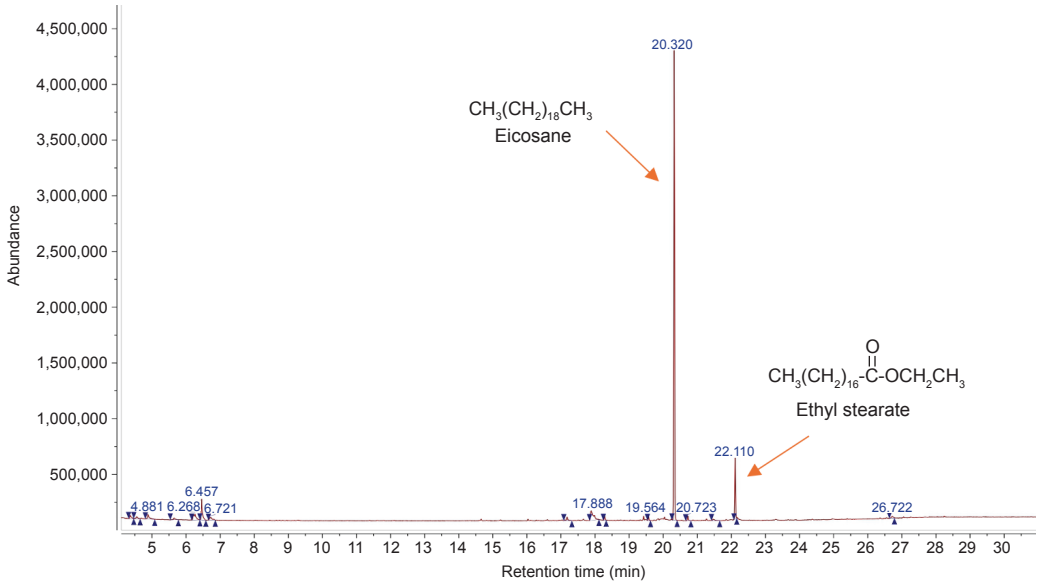
Ethyl stearate is considered a primary metabolite, produced during the logarithmic phase in the bacterium *B. subtilis* strain MN704394.1, essential for its growth. Nevertheless, ethyl stearate may result from a reaction between the fatty acids produced by the bacteria and the ethanol solvent. This aligns with Da Silva et al. (2019) study, which obtained ester compounds with an 80% conversion rate after adding ethanol to the fermentation product containing stearic acid. Further research is required to ascertain whether ethyl stearate originates from bacterial production or is a result of a reaction with the solvent. Nonetheless, the method employed in this study yielded ethyl stearate abundantly and displayed promising antifungal activity.

The primary antifungal mechanism of fatty acids involves their insertion into the lipid bilayer membrane of the fungus, disrupting membrane integrity, which leads to the uncontrolled release of intracellular electrolytes and proteins, ultimately resulting in the disintegration of the fungal cell cytoplasm. Additionally, compounds can penetrate the fungal membrane and interfere with the synthesis of essential components like ergosterol, glucan, chitin, proteins, and glucosamine (Tay & Chong, 2016). Furthermore, exposure to bacterial compounds has been shown to alter fungal morphology, inhibit enzyme activities, and modulate gene expression. One such affected enzyme activity is laccase activity (Schmidt et al., 2015). Laccase, along with other ligninolytic enzymes such as manganese peroxidase and lignin peroxidase, is secreted by white-rot fungi (Yang et al., 2017). *G. boninense* also produces lignolytic enzymes capable of degrading lignin components in plant cell walls, leading to basal stem rot disease in oil palm (Ho et al., 2020).

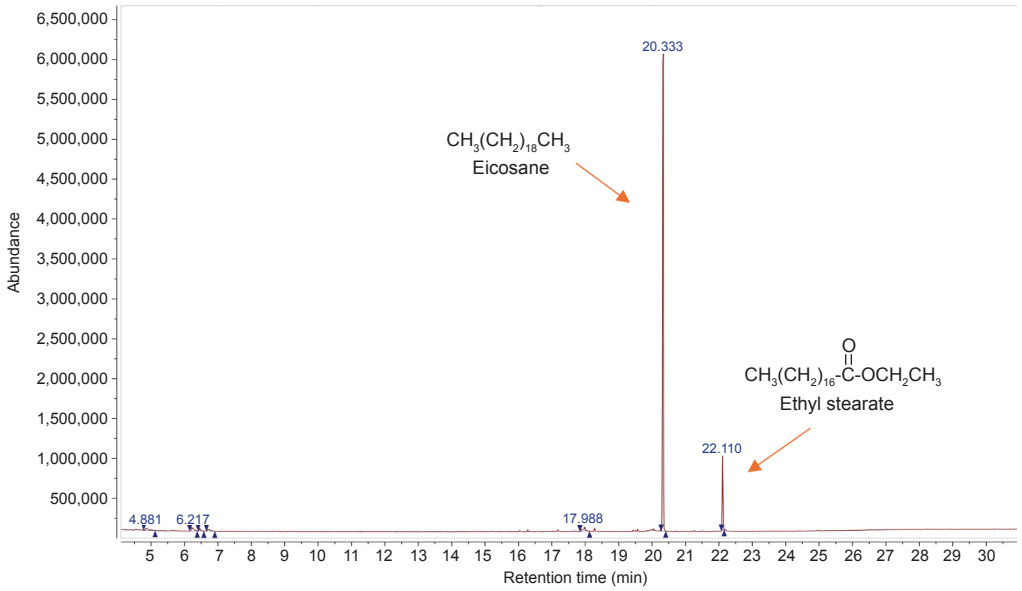
(a)


Abundance
Retention time (min)
20.320
$CH_3(CH_2)_{18}CH_3$
Eicosane
$CH_3(CH_2)_{16}$-C(=O)-OCH_2CH_3
Ethyl stearate
22.109
4.881
6.457
17.887
19.564
23.282

(b)


Abundance
Retention time (min)
20.320
$CH_3(CH_2)_{18}CH_3$
Eicosane
$CH_3(CH_2)_{16}$-C(=O)-OCH_2CH_3
Ethyl stearate
22.110
4.881
6.268
6.457
6.721
17.888
19.564
20.723
26.722

(c)


Abundance
Retention time (min)
20.333
$CH_3(CH_2)_{18}CH_3$
Eicosane
$CH_3(CH_2)_{16}$-C(=O)-OCH_2CH_3
Ethyl stearate
22.110
4.881
6.217
17.988

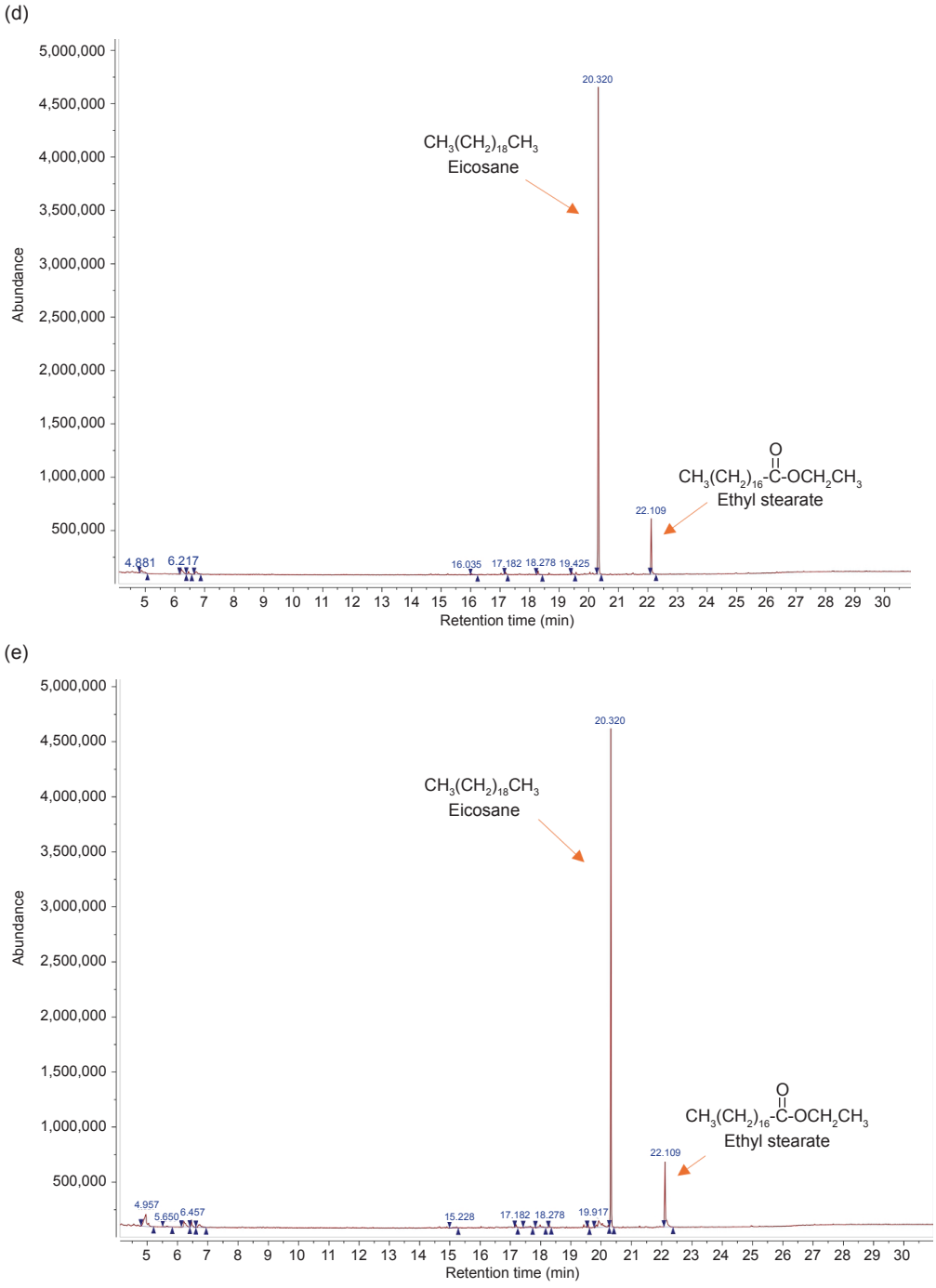


Figure 6. The chromatogram of GC-MS analysis of the ethanol extract at the (a) 8th, (b) 12th, (c) 16th, (d) 20th and (e) 24th hr.

CONCLUSION

The oil palm pathogenic fungus *G. boninense*'s growth can be effectively controlled using *B. subtilis* strain MN704394.1 as a biocidal agent. Ethanol extract from the supernatant culture of *B. subtilis* strain MN704394.1 demonstrated potent antifungal activity, with the highest inhibitory effect (96.44%) observed at the 8th hr when employing the preventive method. GC-MS analysis identified key compounds, including eicosane (68.75% area, retention time 20.32) and ethyl stearate (10.30% area, retention time 22.12), in the crude extract. Eicosane, a long-chain alkane with a high flash point, faces storage challenges at room temperature, limiting its exploration as an antifungal agent. Notably, ethyl stearate, a fatty acid ester, plays a pivotal role in inhibiting *G. boninense*. Utilising *B. subtilis* strain MN704394.1 as a biocidal agent holds significant promise, not only due to its capacity to produce bioactive compounds but also because these compounds exhibit robust antifungal properties.

This approach effectively suppresses *G. boninense*, the causative agent of basal stem rot disease in oil palm plants. The study underscores the potential of harnessing microbial resources for sustainable biological pest control in agriculture, with *B. subtilis* strain MN704394.1 as a promising candidate for eco-friendly fungal disease management.

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