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Research Article

Introducing *Ganoderma* Fungi DNA Fragments into *Spirulina* Algae as a Carrier to Develop Sustainable Biopesticides

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ABSTRACT

The urgency of using biopesticides has been continually emphasised to promote environmental sustainability in line with rising agricultural challenges. The self-DNA concept reveals that DNA fragments from the conspecific species can inhibit their own growth, presenting a promising alternative biopesticide to combat Basal Stem Rot disease caused by *Ganoderma*. *Spirulina platensis*, a promising carrier of DNA, is used in this experiment. This study focuses on introducing *Ganoderma* DNA fragments into Spirulina, referred to as *Ganoderma* introduced *Spirulina* (GIS), and examining the subsequent effects on *Ganoderma* mycelium, including gene-level expression response. We utilised a local strain of *Ganoderma* from the Rejosari Plantation area (Lampung), conducting a simple inhibitory assay by treating *Ganoderma* cultures in Petri dishes with fragmented DNA over a 14-day period. To facilitate the introduction of DNA, 2000 µg and 4000 µg of *Ganoderma* DNA were added, with detection performed using the *GanB2* and *GanB3* markers. An inhibitory test was conducted to observe the response of *Ganoderma* mycelium after GIS treatment, followed by RNA extraction for gene expression analysis. The target genes, including lanosterol synthase (*LS*), cytochrome P450 family 512, subfamily A2 (*CYP512-A2*), and lanosterol 14α-Demethylase (*ERG11*), were analysed using real-time qPCR and relative expression analysis. The results indicate that the introduction of fragmented *Ganoderma* DNA in higher concentrations enhances *Spirulina* growth. It also effectively inhibits *Ganoderma* mycelium growth after GIS treatment, with reduced *ERG11* and *CYP512-A2* expression observed after 14 days. Overall, this research supports the development of biopesticides, promoting sustainable agriculture by protecting soil health and biodiversity.

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INTRODUCTION

In the last decade, the use of organic materials as control agents for plant pests has received enormous attention from researchers and agricultural practitioners worldwide. This relates the issue of the sustainability of the agricultural sector, which is starting to be threatened due to the massive use of chemicals for soil fertility and control of plant pests and diseases (Kumar et al. 2021). The development of biofungicides is crucial for the sustainability of oil palm cultivation, considering environmental safety. Therefore, we initiating a study related to the development of biofungicides based on recent study combining organic materials and DNA fragment (Mazzoleni et al. 2015a; Lanzotti et al. 2022).

In two recent studies, (Mazzoleni et al. 2015) reported the discovery that fragmented extracellular self-DNA (i.e., DNA from members of the same species) exerts species-specific inhibitory effects on plants. In their second study (Mazzoleni et al. 2015b), they documented observations of autotoxicity, meaning that decomposed litter from a plant species specifically inhibited the root growth of its own seedlings. Building on this evidence and earlier theoretical modelling work (Mazzoleni et al. 2007, 2010), researchers proposed a hypothesis that nucleic acids play a role in explaining the species-specific inhibitory effect. DNA extracted from decomposing litter showed significant accumulation of extracellular DNA, particularly in fragments ranging from about 50 to 2000 base pairs. Substrates displaying autotoxic effects were notably rich in DNA fragments of this size. Detailed chemical analysis using ^{13}C NMR further confirmed that only DNA-related signals showed a negative correlation with root growth on conspecific litter. Laboratory experiments validated that purified conspecific DNA inhibited seed germination and root growth specifically when the DNA fragments fell within this size range.

We adopted this concept to formulate a biopesticide for Basal Stem Rot (BSR) disease caused by *Ganoderma* sp. in oil palm field areas. The principle is to combat BSR using *Ganoderma* DNA fragments as a biopesticide, making the approach more sustainable and environmentally friendly. As evidence shows that this disease is spreading rapidly and uncontrollably, compounded by climate change, it is expected that oil palm cultivation will become increasingly unsuitable after 2050, with BSR predicted to reach very high levels across most areas of the island. This shift will likely render palm oil production unsustainable sometime between 2050 and 2100 (Paterson 2019). However, producing DNA fragments of Ganoderma on a large scale is not easy. Thus, we need a carrier to store and deliver the number of extracted fragments, while the carrier also provides various bioactive compound benefits for oil palm.

Macro and microalgae are potential sources of raw materials as DNA carrier in gene delivery technology for developing organic fertilizers and pesticides. Certain transformation techniques, such as cell-penetrating peptides, polymers, liposomes, and metal-organic frameworks, are either newly tested or have yet to be applied in algae. These approaches hold potential for improving the efficiency of delivering protected DNA constructs to algal genomes. The method chosen for a specific alga, it is crucial to assess the necessary infrastructure, availability of molecular tools, expression efficiency, and reproducibility (Gutiérrez & Lauersen 2021). Among various types of microalgae, *Spirulina* (*Artrospira platensis*) is widely studied as a plant biostimulant due to its diverse active ingredients. Spirulina is a blue-green microalga widely cultivated and consumed in China and the United States. Based on the literature, Spirulina is rich in beneficial compounds, including polysaccharides (15-20 %), proteins (60-70 %), carbohydrates, essential fatty acids, vitamins, minerals, chlorophyll, and β-carotene, making it is widely used as nutraceutical food, supplements, and feed (Ai et al. 2023). Recent studies suggest that certain microalgae species can associate with *fragmented* extracellular DNA

(eDNA) and store it as a *natural library*, making it a potential strategic solution in delivering nonself-DNA on an industrial scale. Non-self DNA can enter prokaryotic and eukaryotic organisms naturally or artificially, but maintaining its presence in the natural library needs to be maintained to deliver on target. Like most prokaryotic organisms, *Spirulina* can be transformed to accept extracellular DNA chains, such as plasmids and episomes, in circular and linear forms (Tabakh et al. 2023). Lanzotti et al. (2022) stated that *Spirulina* can take up extracellular DNA naturally. Spirulina introduced with extracellular DNA fragments of *Fusarium oxysporum* showed the best effect on *Lactuca sativa* L. plants as indicated by the lowest mortality and abnormality parameters, with the highest biomass after exposure to *F. oxysporum*. Based on these studies, it is suspected that there is a disease-inhibitory effect by *F. oxysporum* DNA fragments, hereafter known as the self-DNA effect. Meanwhile, the active ingredients in *Spirulina* produce plant health-enhancing effects. The synergistic effect between *Spirulina* and extracellular DNA is expected to be a strategic solution in controlling plant pests and diseases and increasing plant resistance to disease attacks.

In fungi, lanosterol acts as the precursor, directing the flux in biosynthesis ergosterol (*ERG11*) (Lepesheva et al. 2008). Ergosterol, a 5,7-diene oxysterol, is the predominant sterol in fungal cell membranes, playing a key role in controlling membrane permeability and fluidity (Douglas & Konopka 2014). Due to its essential functions, distinctive structure, and specific biosynthetic pathways, ergosterol is the primary target of most clinically used antifungal drugs (Dhingra & Cramer 2017). Ergosterol and related sterols may also be involved in cross-kingdom interactions. In plants, ergosterol acts as a microbe-associated molecular pattern, recognised as an immunologically active "non-self-antigen" (Klemptner et al. 2014). Additionally, glycosylated sterols have been associated with immune protection in animal models of fungal infection (Rella et al. 2015). *CYP51A* is involved in the biosynthesis of Ganoderic acid (*GA*) from lanosterol. P450 enzymes (*CYPs*) from *G. lucidum* expressed in baker's yeast were used to identify key *CYPs* involved in type II GA biosynthesis and to investigate the catalytic sequence of a promiscuous *CYP* (Yuan et al. 2022). Therefore, this study aimed to develop the method of introducing fragmented *Ganoderma* DNA into *Spirulina* (GIS) and to evaluate the molecular response of *Ganoderma* after treatment with GIS.

MATERIALS AND METHODS

Molecular Identification, fragmented DNA preparation and Inhibitory test

First, *Ganoderma* strain obtained from Rejosari plantation area, PTPN 1 regional 7, Lampung, was identified using *GanB3* as the marker. After the strain was confirmed as *Ganoderma boninense* through phylogenetic tree construction, the isolate was cultivated using PDB (potato dextrose broth) medium. After 21-28 days of cultivation, the mycelium was harvested, and whole genome DNA extraction was performed using the Orozco-Castillo et al. (1994) method. The total DNA was then fragmented by incubating it in 95 °C for 30 minutes.

The inhibitory test was conducted in several experiments based on the method of Meitha et al. (2023) with some modifications. Specifically, 100 µL of fragmented *Ganoderma* self-DNA was spread onto PDA, followed by the inoculation of *Ganoderma* with a 1 cm in diameter. We used *Ganoderma* strain with two DNA concentrations, 100 µg mL^{-1} and 800 µg mL^{-1} (five biological replications), with observations made on days 0 (H0), 5 (H5), 8 (H8), 12 (H12), and 15 (H15). Statistical analysis was performed by using one-way ANOVA, with Tukey HSD as the further analysis.

Spirulina Cultivation and *Ganoderma* introduced *Spirulina* (GIS)

Spirulina was cultivated using an Erlenmeyer with a capacity of 5 L. The experiment was conducted indoors using a culture medium of 30 % salinity brine (*spirulina* seedlings: medium, 2:3). Nutrition used modified with TG (*Technical Growth/agricultural fertilizer*) combined with D-(+)- Glucose 300ppm (Himedia, GRM016), CO2 tablets (Tabletica), and Walne medium (Endar et al. 2012). Agricultural fertilisers were applied at a concentration of 40 ppm urea (spur/grow stable phytoplankton species), 30 ppm ZA and 150 ppm KNO_3 (nitrogen source), 12.5 ppm SP-36/TSP (phosphate source), 200 ppm $NaHCO_3$ (carbon source) with pH 8-9.

After successfully cultivated *Spirulina*, we introduced the DNA fragment of *Ganoderma* to the *Spirulina* culture by adding fragmented DNA of *Ganoderma* in some doses (Table 1). *Ganoderma* introduced *Spirulina* (GIS) process was carried out for 14 days with the treatment given by adding fragmented extracellular DNA of *Ganoderma* as follows:

Table 1. Introducing fragmented extracellular DNA (eDNA) *Ganoderma* treatment.

Treatment	Description
C	Control/ without fragmented extracellular DNA
G5	5 mL (2000 µg) fragmented extracellular DNA Ganoderma
G10	10 mL (4000 µg) fragmented extracellular DNA Ganoderma

The culture was continuously aerated with lighting sourced from a 25-watt LED lamp (Recent, SP1000SW). Culture lighting conditions ranged from 5310 lux in the morning, and the highest reached 7022 lux in the afternoon, with pH values ranging from 7-9 and salinity 30-40 %. The humidity and temperature conditions varied, from 64.5 % to the highest, reaching 75 % with temperatures ranging from 25-30 °C. *Spirulina* culture was harvested on day 14 by direct filtering with nylon cloth. Sampling was taken from the medium (liquid), fresh *Spirulina*, and dried *Spirulina*, which was oven-dried at 105° C for 2 hours. Samples were then stored at -4° C for later molecular analysis.

Re-run Inhibitory assay

To evaluate the effect of inhibition of GIS towards *Ganoderma* mycelium, we performed a re-run for inhibitory assay. Two grams of *Ganoderma* introduced *Spirulina* (GIS) from the harvested *Spirulina* C (no eDNA addition), G5 (2000 µg eDNA), and G10 (4000 µg eDNA) were added to PDA medium (10 biological replications), followed by the inoculation of *Ganoderma* with a 1 cm in diameter. Observations were performed over 14 days, with data collection in days 5, 7, 9, 12 and 14. Statistical analysis was performed using one-way ANOVA with Tukey HSD as the further analysis.

DNA Extraction & Validation

DNA extraction was carried out using the Orozco-Castillo et al. (1994) method with some modifications, namely in the lysis and extraction phases using chloroform:isoamyl alcohol (C:I). One gram of sample was mixed with 0.1 gram of PVP and liquid N_2, then ground until smooth. The resulting powder was put into 10 mL of extraction buffer that had been heated and given 1 % β - mercaptoethanol. The mixture was shaken vigorously, then incubated for 30 minutes at 65 °C, and homogenisation using a 1-minute every 10 minutes. The buffered extract solution was allowed to cool at room temperature and then centrifuged at 11,000 rpm for 10 minutes. The supernatant was transferred to a new tube, and the same volume of Chloroform: Isamilalcohol (C:I) solution was added. Then, the solution was shaken vigorously. Centrifugation was carried out for 10 minutes at 11,000 rpm. The upper liquid layer was transferred into a new tube, adding the same volume of cold isopropanol. The mixture

was gently shaken until homogeneous, stored in a refrigerator (4 °C) for 30 minutes, then centrifuged for 10 minutes at 11,000 rpm. The liquid was discarded, and the DNA pellet was dried by inverting the tube. DNA pellets were dissolved in 1 mL of TE buffer, then CH_3 COONa 3 M pH 5.2 as much as 1/10 V and ethanol absolute as much as 2.5 mL. The mixture was shaken until homogeneous and stored in a freezer (-20 °C) for 30 minutes or overnight. The mixture was centrifuged for 10 minutes at 12,000 rpm at 4 °C. The supernatant was discarded, the DNA pellet was washed with 70 % ethanol and then air-dried. DNA pellets were dissolved in 50 mL of TE buffer or ddH_2 O and added 50 mL of RNase 1 mg mL^{-1}, then stored in the freezer. DNA quality was tested by 1.0 % agarose gel electrophoresis, and the concentration was measured using a NanoDrop spectrophotometer at 260/280 and 260/230 nm.

Polymerase Chain Reaction (PCR) & Electrophoresis

PCR was performed using PCR mix from mytaq HS Red Mix (Bioline, Lot No. PM348-B101420) using a T100™ Thermocycler (Biorad, 621BR72483). Amplification of target genes was performed using specific primers (Table 2) by following the protocol of initial denaturation 95 °C 3 min, denaturation 95 °C 30 s, annealing 55 °C (*GanB*) and 40 °C (*SA*) 30 s, elongation 72 °C 1 min, and final elongation 72 °C 5 min (40 cycles). Amplicon visualisation was performed using an electrophoresis instrument by adding 2 % (b v^{-1}) agarose gel added with gel red. Furthermore, visualization was continued with a GelDoc go imaging system (Biorad, serial no. 730BR14249).

RNA extraction and Gene expression analysis

Ganoderma fungi samples were isolated using Chang et al. (1993) method by scraping the mycelium in the surface of agar then ground with liquid nitrogen using a mortar and pestle. The samples were then incubated at 65 °C for 1 hour, with careful inversion every 15 minutes. RNA extraction commenced with 1 volume of chloroform:isoamyl alcohol (C:I), followed by centrifugation. The clear aqueous phase was collected, and an equal volume of phenol:chloroform:Isoamyl alcohol (P:C:I, 1:1:1) was added for re-extraction with C:I in the subsequent step. After collecting the clear phase again, 10M LiCl was added to reach a final concentration of 2M, and samples were incubated at 4 °C overnight.

The following day, samples were centrifuged, and the pellet was resuspended in SSTE buffer, along with 3M sodium acetate (pH 5.2) and absolute ethanol, then incubated at -70 °C for 3 hours. After incubation, the supernatant was discarded, and the pellet was washed with 70 % cold ethanol and air-

Table 2. Primers for *GanB1*, *GanB2*, *GanB3*, *GanB4*, and *SA (Spirulina AnkiMono)* genes.

Primer	Nucleotide (5' to 3')	Size (bp)	Reference
GanB1 Forward Reverse	ATAACGCCTAGAGGAGGGCT GCTTCACTCCGTTTGCTTCG	1750	Present study
GanB2 Forward Reverse	TTACGGCCTACCAAGCGAAC ACGTTTGCGTCTCTGGTCTT	250	Present study
GanB3 Forward Reverse	TAACGGCCTACCAAGCCAAC ACCTTCGTTTCTCAGCGTCA	400	Present study
GanB4 Forward Reverse	ACACTACCAGCAGTCGAGAA ACTCACAAGGCGGAATGGTT	2000	Present study
SA Forward Reverse	AGTAGTCATATGCTTGTCTCAAAGAAT AACACTTCACCAGCACACTCAA	75	(Khaw et al. 2020)

dried. The RNA pellet was dissolved in 30 µL of ddH_2O, and RNA concentration and purity were assessed using a NanoDrop. The resulting RNA was then converted to cDNA with the ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Toyobo, Japan), following the manufacturer's protocol. Finally, cDNA concentrations were verified with a NanoDrop.

Gene expression validation was performed using RT-qPCR (Applied Biosystems Real-Time PCR Instruments). Primers used are listed in Table 3. RT-qPCR conditions were as follows: holding stage at 95 °C for 2 minutes; cycling stage of 45 cycles at 95 °C for 5 seconds, followed by annealing at 55-57 °C for 30 seconds. The melting stage included a step at 95 °C for 15 seconds and a hold at 60 °C for 1 minute.

RESULTS AND DISCUSSION

In this study, we examined the response of *Ganoderma* to fragmented *Ganoderma* DNA treatments at varying doses to validate the Mazzoleni concept at a Petri dish scale. We then introduced *Ganoderma* DNA fragments to Spirulina as a carrier (referred to as *Ganoderma* introduced *Spirulina*, GIS) and observed changes in Spirulina cultivation. Next, we conducted an experiment on *Ganoderma* mycelium colonies in Petri dishes treated with GIS, assessing their response by observing colony inhibition over 14 days. We also performed gene expression analysis to investigate ergosterol biosynthesis and key upstream enzymes related to ergosterol production.

Proof of concept: Inhibitory test of self-DNA Ganoderma

Figure 1A illustrates the inhibition of *Ganoderma* colony diameter (cm) after treatment with fragmented *Ganoderma* DNA at doses of 100 and 800 µg mL^{-1} from day 2 until day 15. Overall, inhibition of *Ganoderma* diameter was observed in the 100 and 800 µg mL^{-1} treatments compared to the control. The colony diameter in the control group gradually increased throughout the period.

In the 100 µg mL^{-1} treatment group, the colony diameter gradually expanded, with the highest growth rate observed on day 8, reaching nearly 6 cm, from just above 2 cm on day 2. After day 8, the colony growth rate slowed, reaching around 7.5 cm by day 15. A similar trend was observed in the 800 µg mL^{-1} treatment group. The colony growth peaked on day 8 at nearly 6 cm and then slowed until day 15, with a final diameter just above 7 cm. Overall, self-DNA doses of 100 µg mL^{-1} and 800 µg mL^{-1} successfully inhibited the growth of *Ganoderma* by 23 % and 26 %, respectively, by day 15.

Table 3. List of primer sequence for gene expression analysis.

Gene target	Direction	Sequences
GBR (House-keeping gene)	Forward	GAGTTCACTGCGGCCGAGTC
	Reverse	TGCAACACGCTTATTCTTCG
Lanosterol synthase (LS)	Forward	CTTCCGCAAGCACTACCCG
	Reverse	AGCAGATGCCCCACGAGCC
Cytochrome P450, family 512, subfamily A2 (CYP512-A2)	Forward	ATGACATCCTCAACTCCCTCG
	Reverse	GACCATCAGCCCCAATACTCT
Lanosterol 14α-Demethylase (ERG11)	Forward	TCTATTCGCACTTGACCACG
	Reverse	TGATCTCATCCTCGATCATGC

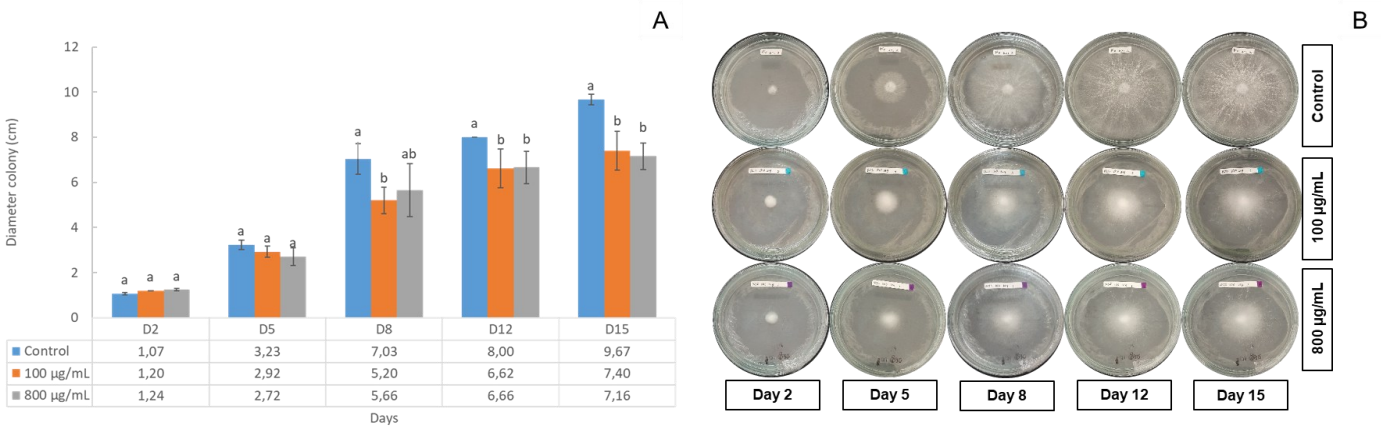

Figure 1. Measurement of *Ganoderma*'s colony diameter after inhibitory assay (A), visualisation of *Ganoderma* after self-DNA treatment (B).

Visualisation of *Ganoderma* mycelium growth from days 0 to 15 is shown in Figure 1B.

The growth response of Spirulina after introduced by fragmented DNA of Ganoderma

The culture condition of *Spirulina* with the addition of fragmented *Ganoderma* DNA can be seen in Figure 2. On day 0, uniform *Spirulina* growth was visually observed in both control (C), 5 mL of 400 µg mL^{-1} (2000 µg) (G5) and 10 mL of 400 µg mL^{-1} (4000 µg) (G10) *Ganoderma* eDNA treatments. On day 3, *Spirulina* cultures began to show differences in biomass density, as indicated by differences in the green-blue colour of the treatments compared to the control. By day 3, it was already apparent that the growth of *Spirulina* in the G10 treatment was faster than that of G5 and control group. By day 7, *Spirulina* biomass density was visually greater in treatment G10, followed by G5 and control group. Based on visual observation, it can be seen that the fragmented DNA *Ganoderma* treatment affected the growth pattern of *Spirulina* biomass. Based on observations, it was suspected that fragmented DNA *Ganoderma* accelerated the growth rate of *Spirulina*, where the addition of 10 mL fragmented DNA *Ganoderma* showed a higher growth acceleration effect.

The fragmented DNA *Ganoderma* is considered to supply nutrients to *Spirulina* and synergistically affect its growth. It is known that DNA fragments consist of three main constituents, namely nitrogenous bases, phosphate, and deoxyribose sugar, so it is thought to play a positive role in growth. However, Palomba et al. (2022) reported that adding non-self DNA to microalgae cultures of *Chlamydomonas reinhardtii* and *Nannochloropsis gaditama* did not affect growth. In contrast, self-DNA freshwater and marine microalgae, *C. reinhardtii* and *N. gaditama*, showed growth inhibition respectively. Additionally, there was a phenotypic effect in the form of cell damage with the formation of heteromorphic (abnormal) aggregates of cell palmelloids embedded in the extracellular matrix. In contrast, this phenomenon was not observed in nonself-DNA exposure.

Validation of DNA Extraction and PCR of Spirulina and Ganoderma markers

To validate the existence of *Ganoderma*'s DNA in the *Spirulina* cultivation and to prove that fragmented DNA *Ganoderma* is incorporated into *Spirulina*, DNA extraction was performed on the filtrate (containing only the *Spirulina* medium) and residue of fresh and dried harvested *Spirulina*. DNA amplification was performed for both *Spirulina* and *Ganoderma* DNA using specific primers.

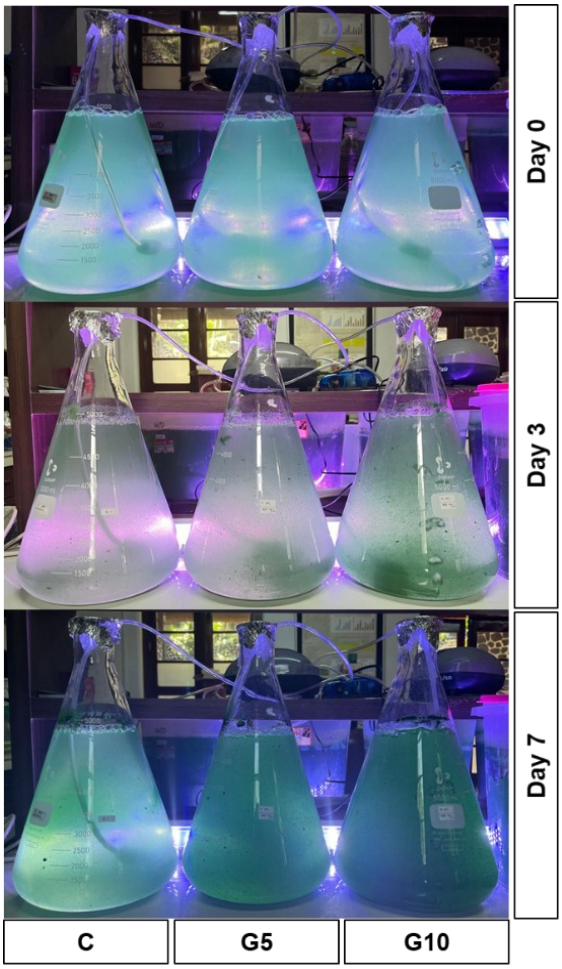

Figure 2. Visualisation response of fragmented DNA *Ganoderma* introduced to cultivation condition of *Spirulina*. C = non-treated *Spirulina*/ Control, G5 = *Spirulina* treated by 5 mL of Fragmented DNA of *Ganoderma*, G10 = *Spirulina* treated by 10 mL of Fragmented DNA of *Ganoderma*.

First, to confirm the existence of *Spirulina* genomic DNA, quantity and quality data were obtained from fresh and dried harvested *Spirulina* as shown in Table 4. Prior to this, the genomic DNA of each sample was run to confirm the successful DNA isolation (Figure 3). The quality of *Spirulina* genomic DNA in fresh and dried conditions was also optimised. Fresh and dried *Spirulina* DNA in each treatment showed considerable concentrations ranging from 1962.35 to 2224.40 ng μL^{-1}. The light absorption ratios at 260 and 280 nm wavelengths ranged from 1.72 to 2.09. Simultaneously, the absorption ratio with wavelengths of 260 nm and 230 nm ranged from 1.43 to 2.15. According to Psifidi et al. (2015), a 260/230 nm wavelength ratio of ≥ 1.8 indicates that the extracted genomic DNA is free from carbohydrate contamination.

According to (Khaw et al. 2020), validation of successfully isolated *Spirulina* DNA was amplified using the *SA* (*Spirulina AnkiMono*) gene. The electropherogram results confirmed that each sample was *Spirulina* DNA, as indicated by a band approximately 75 bp (Figure 4).

Secondly, to confirm the presence of *Ganoderma* DNA in *Spirulina* cultivation after GIS process, detection was performed for *Ganoderma* DNA in the filtrate (containing only the *Spirulina* mediums), and residues of fresh and dried harvested *Spirulina* using four *Ganoderma* markers: *GanB1*, *GanB2*, *GanB3*, and *GanB4*.

In the filtrate phase, it was found that none of the *Ganoderma* DNA was detected (Figure 5). It is suspected that DNA fragments do not remain in the medium or are available only in relatively small amounts. In fresh residue of *Spirulina*, *GanB2* and *GanB3* successfully amplified in G5 and G10 treat-

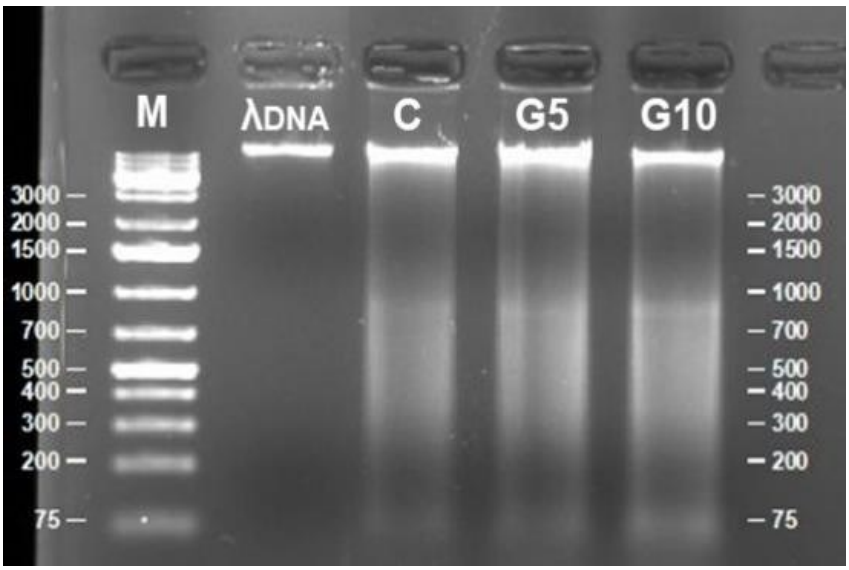

Figure 3. Electropherogram of genomic *Spirulina* DNA. Notes: M = 1kb Ladder, λDNA = DNA Marker, C = non-treated *Spirulina*/ Control, G5 = *Spirulina* treated by 5 mL of Fragmented DNA of *Ganoderma*, G10 = *Spirulina* treated by 10 mL of Fragmented DNA of *Ganoderma*.

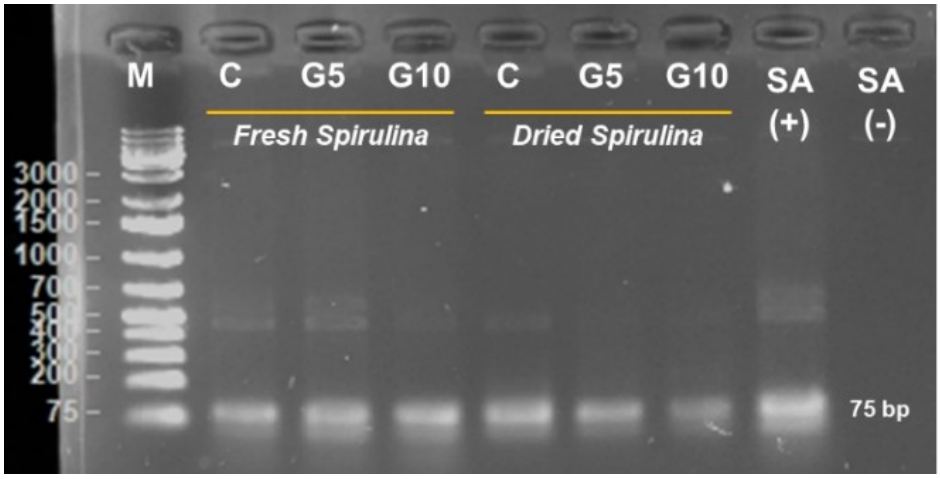

Figure 4. Electropherogram of *Spirulina* DNA using *Spirulina* Ankimono (SA) Marker. Note: M= 1kb Ladder, C = non-treated *Spirulina*/ Control, G5 = *Spirulina* treated by 5 mL of fragmented DNA of Ganoderma, G10 = *Spirulina* treated by 10 mL of fragmented DNA of *Ganoderma*, SA (+)= confirmed Spirulina DNA, SA(-)= negative control of SA.

ments, which are the addition of 5 ml (2000 μg) and 10 ml (4000 μg) of fragmented *Ganoderma* DNA, respectively. Based on the electrophoresis results, the amplicons in the G10 treatment were thicker than G5. *GanB1* and *GanB4* were not detected in any samples. This thought to be due to their larger sizes, nearly 2000 bp, making them more easily damaged when introduced into *Spirulina* culture for 14 days. The thickest *GanB2* and *GanB3* gene bands in *Spirulina* G10, followed by *Spirulina* G5 and C, confirm that the addition of more *Ganoderma* DNA was suggested to be in line with the abundance of DNA fragments that *Spirulina* can take up. DNA fragments were not detected in the liquid medium but in the fresh *Spirulina* biomass. This data led to the hypothesis that the *Ganoderma* DNA fragments were successfully taken up naturally, either attached to the membrane or into the cell.

However, *GanB2* and *GanB3* gene amplicons in dried *Spirulina*-harvested showed relatively uniform thickness DNA bands, while *GanB1* and *GanB4* were not detected (Figure 6). The fragment sizes of *GanB1* and *GanB4* are approximately 1750 and 2000 bp, respectively. Since the DNA genome was fragmented into pieces smaller than 1000 bp, *GanB1* and *GanB4* could

Table 4. Quantity and quality of *Spirulina* Genomic DNA.

Type of Sample	Treatment	DNA Concentration (ng μL^{-1})	Purity (λ260/280)	Purity (λ260/230)
Fresh Spirulina	C	2224.40	2.06	1.95
	G5	1980.23	2.08	2.07
	G10	2114.00	2.09	2.15
Dried Spirulina	C	1968.45	1.76	1.49
	G5	1962.35	1.72	1.43
	G10	2044.55	1.81	1.52

Note: C = non-treated *Spirulina*/ Control, G5 = *Spirulina* treated by 5 mL of Fragmented DNA of *Ganoderma*, G10 = *Spirulina* treated by 10 mL of Fragmented DNA of *Ganoderma*

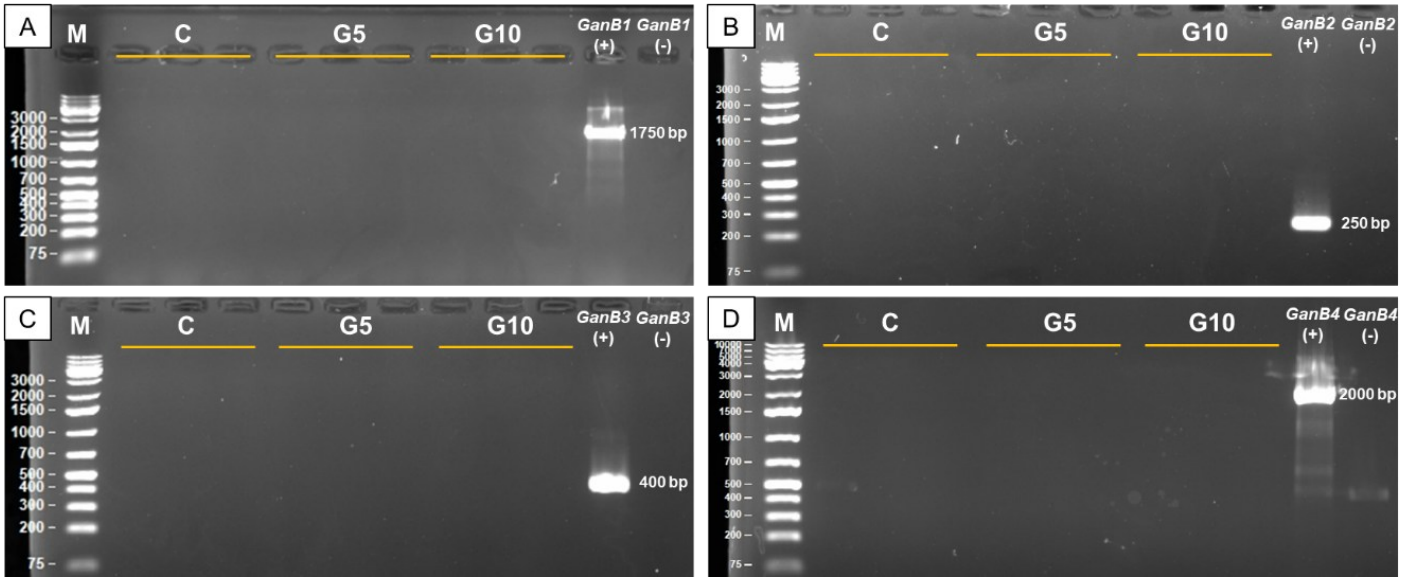

Figure 5. Electropherogram of *Ganoderma* marker in filtrate medium. Notes: A = *GanB1*, B = *GanB2*, C = *GanB3*, D = *GanB4*, M = 1kb Ladder, C = non-treated *Spirulina*/ control, G5 = *Spirulina* treated by 5 mL of fragmented DNA of *Ganoderma*, G10 = *Spirulina* treated by 10 mL of fragmented DNA of *Ganoderma*.

not be amplified. Conversely, oven-drying might cause vaporisation and transfer of genetic material. This is reinforced by the *GanB2* and *GanB3* bands in treatments G5 and G10, which significantly reduced thickness. Drying of *Spirulina* biomass serves to preserve the sample and detect the consistency of the fragments. Therefore, optimising the drying process to preserve the *Ganoderma* DNA fragments and minimise genetic material transfer is necessary.

Another batch of experiment was conducted to show the reproducibility of previous results in fresh *Spirulina* samples. Figure 7A shows that the GanB2 marker in sample G10 consistently produces the thickest band, followed by G5. However, control group sample shows a thin band in the same size. Contrary to previous electropherogram results, the *GanB3* amplification results show relatively the same band thickness in all treatments, including the control. Therefore, *GanB2* is a marker consistently showing the abundance of *Ganoderma* DNA fragments according to the dose. Hilmi et al. (2022) performed PCR assays to detect *Ganoderma* in oil palm plantlets. They found that real-time PCR assays are more sensitive and rapid in *Ganoderma* detection compared to conventional PCR and culture-based assays.

Physiological effect and gene expression assay after Ganoderma introduced to spirulina (GIS) treatment on Ganoderma colony

Subsequently, we re-examined the physiological effects and conducted targeted gene expression analysis of *Ganoderma* mycelium colony after GIS treatment for 14 days. This experiment mimics the future condition of *Ganoderma*

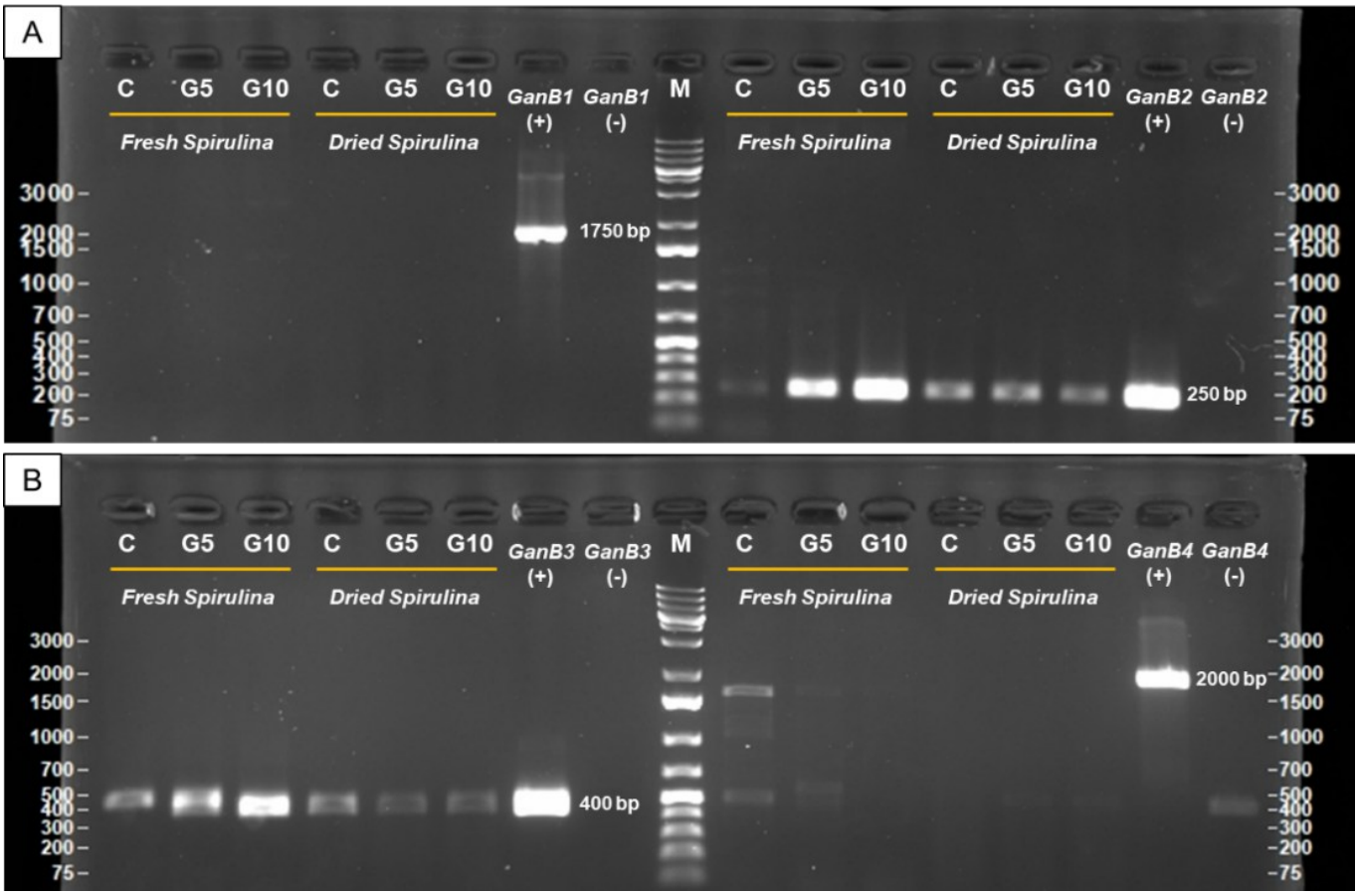

Figure 6. Electropherogram of *Ganoderma* DNA detection in fresh and dried *Spirulina* using *Ganoderma* genes marker *GanB1* & *GanB2* (A), *GanB3* & *GanB4* (B). Notes: M = 1kb Ladder, C = non-treated *Spirulina*/ Control, G5 = *Spirulina* treated by 5 mL of Fragmented DNA of *Ganoderma*, G10 = *Spirulina* treated by 10 mL of Fragmented DNA of *Ganoderma*.

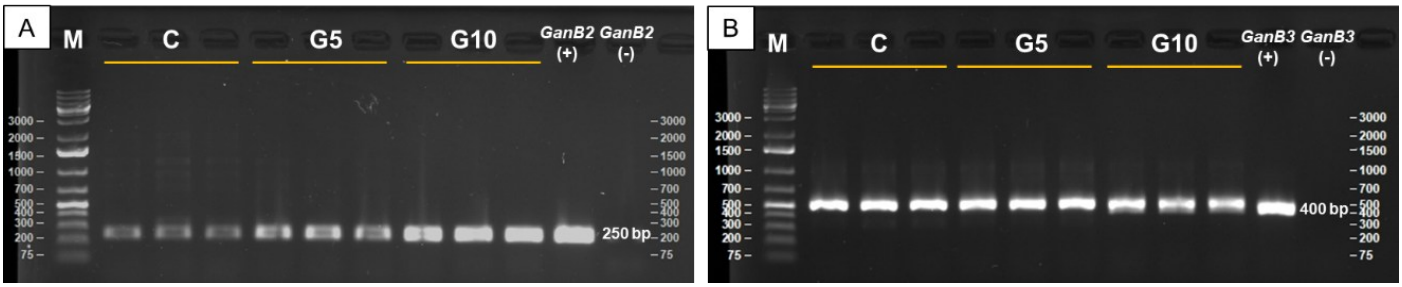

Figure 7. Electropherogram of *Ganoderma* DNA detection in fresh *Spirulina* using *Ganoderma GanB2* (A) and *GanB3* (B) marker. Notes: M = 1kb Ladder, C = non-treated *Spirulina*/ control, G5 = *Spirulina* treated by 5 mL of Fragmented DNA of *Ganoderma*, G10 = *Spirulina* treated by 10 mL of Fragmented DNA of *Ganoderma*.

fruiting bodies in oil palm field after GIS treatment. The assessment involved measuring mycelium colony diameter and analysing gene expression of three target genes, including Lanosterol synthase (*LS*), Cytochrome P450, family 512, subfamily A2 (*CYP512-A2)*, and Lanosterol 14α-Demethylase (*ERG11)*.

Figure 8A and 8B depict the *Ganoderma*'s colony inhibition after GIS treatment at 2.000 µg (G5) and 4.000 µg (G10) doses for 14 days observation compared to control group. Treatment of G5 showed the highest inhibition of the *Ganoderma*'s colony diameter compared to G10 and control group. In addition, *ERG11* in G5 treatment shows similar expression level with control group with no GIS treatment, and higher than G10. Additionally, CYP512-A2 expression was low, indicating that the lanosterol precursor was fully directed towards ergosterol production. Thus, GIS treatment at 2.000 µg dose successfully inhibited the mycelium growth by reducing *ERG11* biosynthesis.

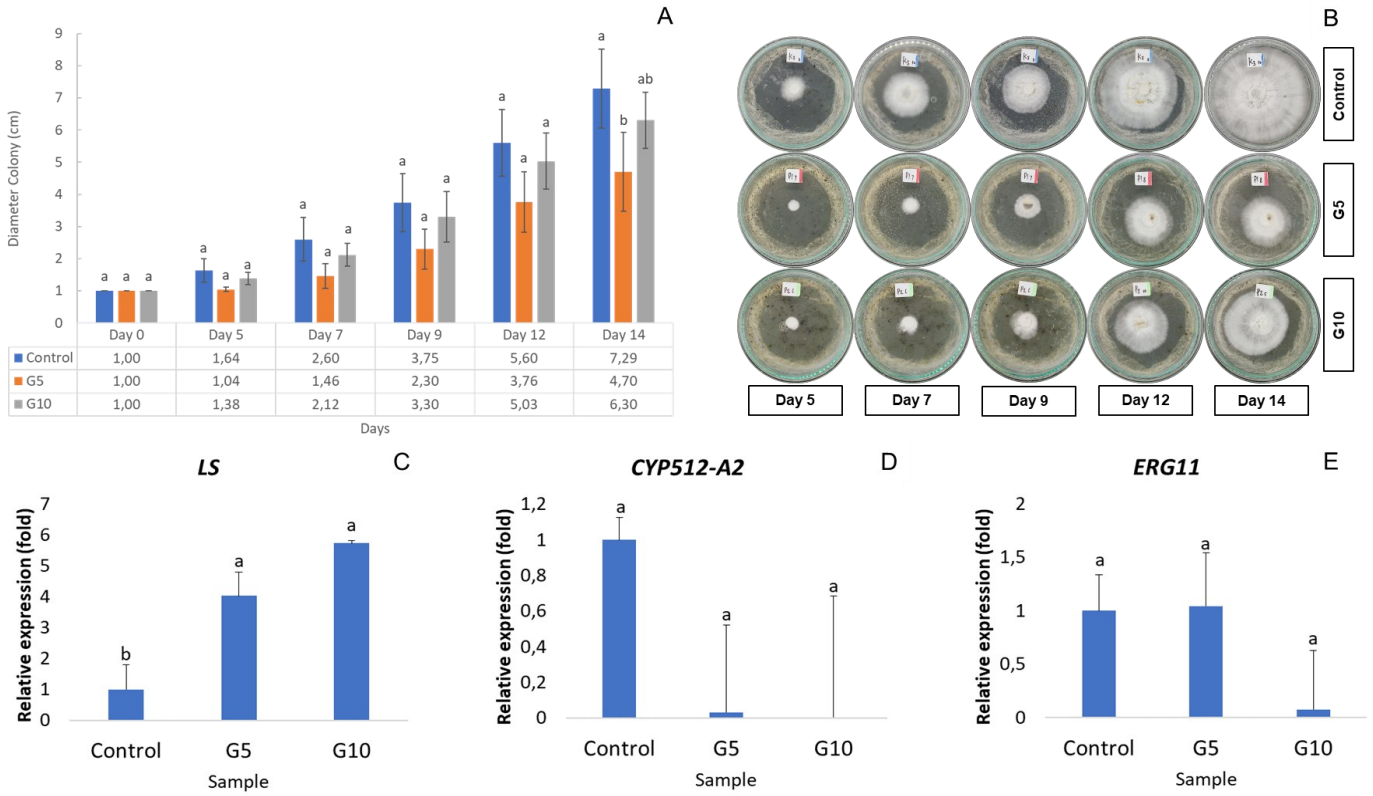

Figure 8. *Ganoderma*'s colony diameter after re-run inhibitory assay (A), visualisation of *Ganoderma*'s colony inhibition after *Ganoderma* Introduced Spirulina (GIS) treatment (B), expression level of three gene targets related to *Ganoderma* mycelium growth: *LS* (C), *CYP512-A2* (D), *ERG11* (E). Note: Control = non-treated *Spirulina*, G5 = *Spirulina* treated by 5 mL of Fragmented DNA of *Ganoderma*, G10 = *Spirulina* treated by 10 mL of Fragmented DNA of *Ganoderma*

CONCLUSIONS

This study successfully demonstrates the introduction of fragmented *Ganoderma* DNA, evidenced by the high growth rate of *Spirulina* at the highest DNA concentration. Additionally, *Ganoderma* DNA was detected in fresh *Spirulina* using the *GanB2* and *GanB3* markers. The *GanB2* marker showed a thicker band at higher concentrations (10 mL or 4000 µg) of fragmented *Ganoderma* DNA introduced into *Spirulina*. Moreover, 5 mL of *Ganoderma* introduced *Spirulina* (GIS) inhibited *Ganoderma* mycelium colony growth at the Petri dish scale, supported by a low expression level of ergosterol (*ERG11*) after 14 days of treatment. This is the first result demonstration of Spirulina as a carrier for environmental DNA delivery for future bio fungicides.

AUTHOR CONTRIBUTION

TA.P.R and R.A.P designed the research and supervised the entire process. D.D.E, D.N.K, A.A.A and TA.P.R collected and analysed the data. M.A.A and G.W.P wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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