

In Vitro Effect of Indigenous Antagonistic Microorganisms of Dibombari Oil Palm Plantation on in *Ganoderma sp.*, Principal Agent of Basal Stem Rot of Oil Palm (*Elaeis guineensis* jacq)

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Abstract

Ganoderma sp. is the main fungus that affects the oil palm, causing the basal stem rot. This fungus can lead to a significant economic loss of 43% in an oil palm plantation. To address this challenge, the management strategy focuses on developing a cost-effective and environmentally friendly integrated control method. Special emphasis has been placed on the use of indigenous antagonistic microorganisms. To achieve this, the microbial biodiversity in oil palm has been explored. Fungi and bacteria have been isolated from soil beneath their roots. Additionally, *Ganoderma sp.* has been isolated from fruiting bodies located at the base of infected oil palms. The antagonistic effect of these microorganisms on *Ganoderma sp.* has been assessed *in vitro*. The results showed that in dual culture test with *Ganoderma sp.*, two microorganisms, the fungus (CS1) and the bacterium (IBS6) showed fungicidal effects with PIRG value of 66% and 72% respectively. Furthermore, the molecular identification revealed that fungus (CS1) exhibits a 99.79% similarity with *Trichoderma virens*, while the bacterium (IBS₆) shows 100% similarity with *Bacillus subtilis*.

Keywords

Antagonistic, *Bacillus subtilis*, *Ganoderma sp.*, Microorganisms, *Trichoderma Vierns*

1. Introduction

Oil palm (*Elaeis guineensis* jacq) is a monocotyledon from the family of Arecaceae. It grows in the tropical areas, mainly in gulf guinea. It is an economically important cash crop, grown primarily for its oil, and has become one of the main crops in the world. To date, oil palm is the world's highest-yielding oil-producing crop. Unfortunately, one of the major constraints faced by oil palm farmers is the devastating disease known as *Ganoderma* disease, caused by *Ganoderma sp.* The fungus was reported before on older palms, but now it has been detected on younger palms aged 1 - 2 years old [1]. *Ganoderma* disease not only causes reduction in total oil yield but also results in direct loss of stands due to palm death and economic losses.

Despite the effectiveness of synthetic chemicals in plant disease management, their continuous utilization has resulted in the development of new streams of fungi with multiple resistances, which further complicates the management of plant diseases [2]. Furthermore, the use of these chemicals in plant protection is also being restricted because of their residual toxicity, long degradation period, environmental contamination, chemical residues in food materials [3].

Therefore, there is an increasing concern and effort to develop a better plant disease management approach that is not only safe and ecological friendly but also economically feasible for farmers. The use of indigenous antagonistic microorganisms agents could provide better compliance with these guidelines. These indigenous microorganisms are successful colonizers of their habitats due to their efficient utilization of available substrates and production of antibiotics and enzymes [4].

The aim of this study is to select the antagonistic indigenous microorganisms isolates from rhizospheric soil and roots of oil palm and evaluate their potential biological control against *Ganoderma sp. in vitro*.

2. Materials and Methods

2.1. Sampling and Isolation of *Ganoderma sp.*

The fruiting body of *Ganoderma sp.* located at the base of the oil palm stem was collected using a sterile knife, then transported to the Plant Biology Laboratory of the University of Douala.

These fruiting body samples were cleaned with the tap water using a brush, the external part was removed, and the internal structures were cut into cubes. These fragments surface were sterilised with 70% alcohol for 30 seconds, then rinsed twice with sterile distilled water. The samples pieces were inoculated into petri dishes (9 cm diameter) containing 15 ml of PDA medium [5]. Incubation was carried out in darkness at 27°C. Data were recorded every daily over a 4-weeks period. Fungal samples were subjected to successive subcultures until pure mycelium was obtained.

2.2. Sampling of Soil and Roots of Oil Palm

2.2.1. Soil

Soil samples were collected from 4 different blocks (A3/06; B4/74; D4/74; G7/75)

of socapalm Dibombari (Littoral-Cameroon) Plantation where the disease incidence is high. Within each block, 4 asymptomatic plants located near disease foci were selected. An 800 g soil samples was collected within 1 meter of the root system of each plant in the 4 cardinal directions. Before each sampling, the soil surface was cleaned of leaves and other organic debris. The soil samples were collected from the 30 - 40 cm soil layer [6], carefully mixed, and sent to the laboratory.

2.2.2. Roots

Roots sampling was conducted in 4 different blocks (Rao. 2018) of socapalm dibombari with high disease incidence. For each block, one asymptomatic oil palm located in the disease foci was selected. Roots were collected aseptically at a depth of 0.2 - 20 cm [7]. The roots were stored with rhizospheric soil in bags at 4°C until use to prevent drying out and preserve the integrity of microorganisms associated with the root.

2.3. Isolation of Microorganisms from Soil and Roots

2.3.1. Soil Microorganisms

The collected soil samples were crushed and sieved, the microorganisms were isolated according to the method of [6], which consisted of taking 10 g of crushed sample and transferring it to 90 ml of sterile distilled water, then shaking for 30 minutes, which constituted a 10^{-1} dilutions. From that solution, other solutions were prepared by transferring 1 ml of solution into 9 ml of sterile distilled water up to 10^{-8} [8]. Only dilutions 10^{-4} , 10^{-5} , 10^{-6} and 10^{-7} were used for inoculation of the solid medium [9].

Inoculation consisted of taking 0.1 ml of each dilution using a pipette and depositing it into a petri dish containing the culture medium; three replicates were performed for each dilution.

2.3.2. Roots' Microorganisms

Roots samples were washed with tap water, cut into 2 cm pieces, disinfected with 70% ethanol for 30 seconds, and rinsed three times with distilled water to remove any residual disinfectant. The pieces were then cultured on PDA medium in petri dishes and incubated in the dark for 7 days for fungal growth [6] and 2 days for bacterial growth.

2.4. Microorganisms Purification

2.4.1. Bacteria

Bacteria that grew in the petri dishes were subcultured and purified several times by streaking on PCA medium. The method used was the quadrant technique [10].

2.4.2. Fungi

Colonies showing fine filaments (hyphae) in the incubated petri dishes were identified as fungi and were isolated and subcultured using a sterilized platinum loop on the PDA medium. Incubation was performed at 28°C for 4 to 6 days. This

process was repeated until pure strains were obtained [11].

2.5. Screening of Isolated Microorganisms

Direct Confrontation

All isolated microorganisms underwent a direct confrontation test to assess their competitiveness against the pathogen responsible for the disease [12]. This test identifies the most competitive microorganism showing potential for use in biological control.

1) Fungi

This technique consists of placing a *Ganoderma sp.* mycelium disk and an antagonistic disk to be tested in a diametrically opposite position in the same 90 mm diameter petri dish containing PDA medium. The two 6 mm mycelium disks of the same age (4 days old) were placed about 30 mm apart from each other relative to the center of petri dish [12]. The control consisted of placing a 6mm diameter of *Ganoderma sp.* (4 days old) alone at the center of the petri dish containing PDA culture medium.

Incubation was performed at 27°C for 7 days with daily observations. After 3 days of incubation, the percentage of inhibition of diameter growth (PIDG) of the pathogen was assessed using the method of [13]. Three repetitions were made per microorganisms.

$$\text{PIDG (\%)} = (1 - C_n/C_o) \times 100$$

C_o = control fungi diameter

C_n = fungal diameter confronted by the antagonistic microorganism

2) Bacteria

The direct confrontation assay was conducted in Petri dishes containing PDA medium. A 6 mm diameter mycelial disk of *Ganoderma sp.* (4 days old) was placed at the center of the petri dish surrounded by two streaks of bacterial (2 days old) culture spaced 2 cm apart. Three replicates were performed for each confrontation. The dishes were incubated at 27°C in darkness for 7 days and observed daily. The control consisted of monoculture of *Ganoderma sp.* Inoculated at the center of the petri dish. The results were evaluated by measuring the distance of mycelial growth of *Ganoderma sp.* towards the bacterial antagonist, and the percentage of inhibition was calculated using the equation reported by [14].

$$\text{(\% Inhibition)} = (1 - D_n/D_o) \times 100$$

D_o = control fungi diameter

D_n = fungal diameter affected by the bacteria

2.6. Characterization and Identification of Antagonistic Microorganisms

2.6.1. Fungi

1) Morphological description

The macroscopic appearance of fungi was evaluated from cultures on PDA medium, and the following characteristics were examined:

- thallus color and texture;
- underside color of colony;
- mycelial growth rate.

2) Microscopic description

The method consisted of cutting a small piece of scotch tape and applying it with its adhesive side to the colony using tweezers, then placing it on a clean slide. Observations were made at 40× magnification, and the following criteria observe:

- spore morphology;
- mycelium characteristic: hyphal color and size;
- branching pattern, clamp connection.

2.6.2. Bacteria

1) Macroscopic observations of colonies

The initial step in bacterial strain identification is the macroscopic examination of isolated colonies. The main characteristic evaluated are: shape, color, elevation, contour, size, surface, and opacity [15].

2) Microscopic description

a) Biochemical characterization

i) Catalase test

It involves the search for catalase, an important enzyme for the elimination of hydrogen peroxide according to the following reaction:

In bacteriology, the catalase test is used to identify and characterize bacteria, particularly Gram-positive bacteria. The test consisted of transferring a bacterial colony from a solid agar plate to a sterile slide, followed by the addition of a drop of hydrogen peroxide (H₂O₂). The presence of catalase is demonstrated by the immediate evolution of gas bubbles [16].

ii) Oxydase test

The oxydase test is a diagnostic tool used to detect the presence of oxydase enzyme in bacteria [17]. This test is crucial for identifying Gram-negative bacteria, as it helps to differentiate oxidase-positive bacteria, such as *Pseudomonas* species, and oxidase-negative bacteria [18].

The oxydase test involves adding a reagent typically tetramethyl-P-Phenylenediamine, to a bacterial colony [18]. If the bacteria possess the oxydase enzyme, the reagent will be oxydized and change color. The oxydase test results are interpreted as follow:

Positive result: bacterium is oxydase positive, indicating the presence of the oxydase enzyme, and the reagent changes color.

Negative result: the bacterium is oxidase-negative indicating the absence of oxydase enzyme, and the reagent does not change the color.

2.6.3. Molecular Identification

Genomic DNA was extracted from the samples received using the Quick-DNA™ Fungal/Bacterial kit (Zymo Research, Catalogue No. D6005). The ITS target region was amplified using OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue

No. M0486) with the primers presented in **Table 1** for fungi and **Table 2** for bacteria. The PCR products were run on a gel and cleaned up enzymatically using the EXOSAP method. The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were analyzed on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific) for each reaction for every sample, as listed in Section 1. DNASTAR was used Genomic DNA was extracted from the samples received using the Quick-DNA™ Fungal/Bacterial kit (Zymo Research, Catalogue No. D6005). The ITS target region was amplified using OneTaq® Quick-to analyse the ab1 files generated by the ABI 3500XL Genetic Analyzer and results were obtained by a BLAST search (NCBI).

Table 1. ITS Primers sequences.

Name of primer	Target	Sequence 5' to 3'
ITS-1	rDNA sequences	TCCGTAGGTGAACCTGCG
ITS-4	rDNAsquences	TCCTCCGCTTATTGATATGC

Table 2. ITS Primers sequences.

Name of primer	Target	Sequence 5' to 3'
ITS 16S-27F	16S rDNA sequence	AGAGTTTGATCMTGGCTCAG
ITS 16S-1492R	16S rDNA sequence	CGGTTACCTTGTTACGACTT

2.7. Statical Analysis

Data analysis was performed using SAS (Statistical Analysis System) software for statistical analyses. The result of qualitative and quantitative variables were expressed as percentages and illustrated using bar/ graphs generated with microsoft Excel. For the isolated microorganisms, analysis of variance (ANOVA) was conducted. To compare average values of groups student's t-test, Newman and Keuls tests were performed at a significance level of 5%.

3. Results

3.1. Sampling, Isolation and Purification of *Ganoderma sp.*

The fruiting body of *Ganoderma sp.* located at the base of the oil palm tree stem (**Figure 1**) has a semi-circular shape, with a shiny color ranging from orange to reddish-brown coloration, and its fresh is colored and woody (**Figure 2**).

3.1.1. Morphologic Characteristic of Mycelium

A 14-day-old purified mycelium of *Ganoderma sp.* exhibits a development of dense, white, cottony filaments with a thin texture. The aerial filaments are abundant and slightly orange at the edge (**Figure 3**). This mycelium has a moderate

average growth. After 6 days, the filament reaches the edge of the 9 cm diameter petri dish.



Figure 1. Fruiting body located at the base.



Figure 2. Fruiting body harvested.



Figure 3. *Ganoderma sp. mycelium.*

3.1.2. Microscopic Characteristics

Microscopic examination reveals that the hyphae of isolated *Ganoderma sp.* are branched, forming a dense and complex network with secondary and tertiary branches. The hyphae are septate and narrow, measuring 5 - 15 μm in width, and display clamp connections (Figure 4).

Spoores are spherical

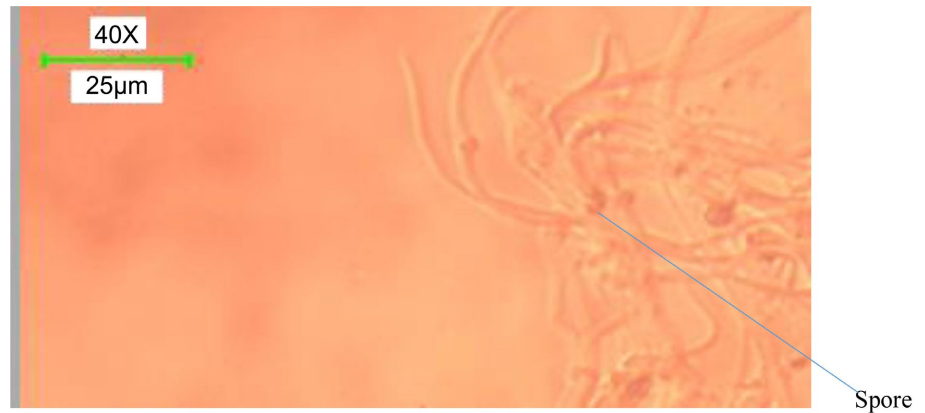


Figure 4. *Ganoderma sp* hypha.

3.2. Isolation and Purification of Microorganisms

The search for microorganisms from the rhizospheric soil of the palm grove and oil palm roots resulted in the isolation of 2 types of microorganisms: 14 fungal isolates (CR₁, CR₂, CR₃, CR₄, CR₅, CR₆, CR₈, CS₁, CS₂CS₄, CR₁₀, CR₉, CS₁₁, CS₁₂) and 16 bacterial isolates (IBS₁, IBS₂, IBS₃, IBS₄, IBS₅, IBS₆, IBS₇, IBS₈, IBS₉, IBS₁₀, IBS₁₁, IBS₁₂, IBR₁, IBR₂, IBS₁₃, IBR₄). CR refers to root-associated fungi, CS to soil-associated fungi, IBS to soil-associated bacteria and IBR to root-associated bacteria. Fungi dominate root isolates for 64% compared to 36% for bacteria. However, these proportions are reversed in soil where bacteria account 75% and fungi 25% (Figure 5).

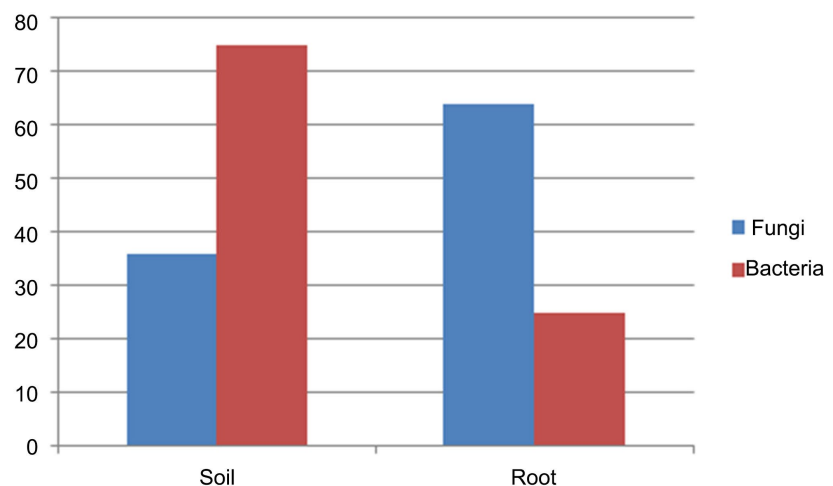


Figure 5. Diversity and distribution of microorganisms in the oil palm ecosystem.

In Vitro Antagonism Assay

1) Direct confrontation

The various microorganisms isolated were screened for their antifungal activity. Two microorganisms gave a PIRG value more than 50%. The fungus (CS₁) and the bacterium (IBS₆) showed significant antifungal activity, inhibiting pathogen growth by 66% and 72%, respectively (Figure 6, Figure 7). On control plates, colony of *Ganoderma sp.* covered the 9 cm diameter of petri dish in 6 days.

In direct dual culture with fungus (CS₁), *Ganoderma sp.* exhibits growth similar to the negative control during the first 3 days. However, starting from the 3rd day, its growth slows down, reaching a maximum diameter of 40 mm on the 4th, followed by a reduction to 30 mm (Figure 8). The hyphae of the phytopathogen swelled up, and the spores of the antagonistic fungus (CS₁) were observed on it, followed by its rupture (Figure 9).

In direct confrontation with the IBS₆ bacterium, *Ganoderma sp.* growth is slow from the early days compared to the control. It reaches its maximum on the 5th day with a radial growth diameter of 25 mm and then stabilizes. No physical contact was observed between the bacterium IBS₆ and the pathogen *Ganoderma sp.* (Figure 10).

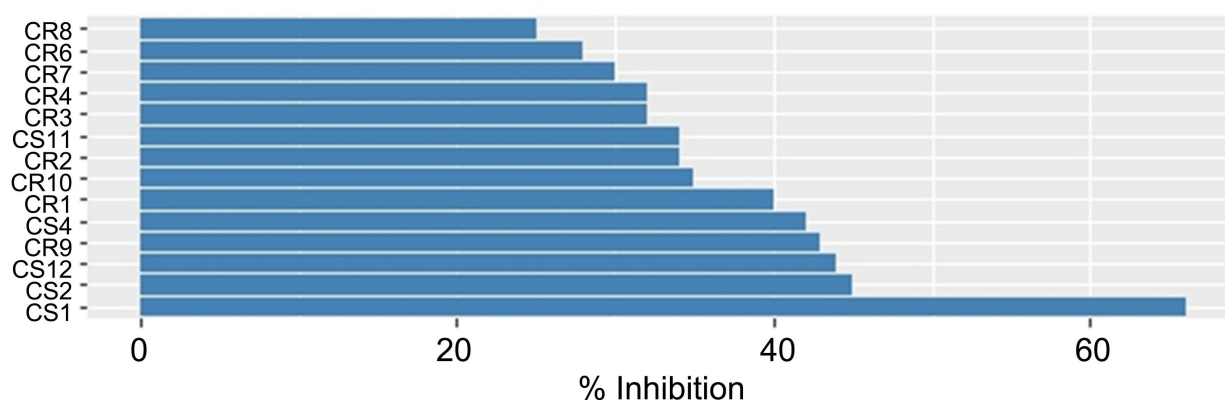


Figure 6. Percentage of inhibition of *Ganoderma sp.* induced by isolated fungi.

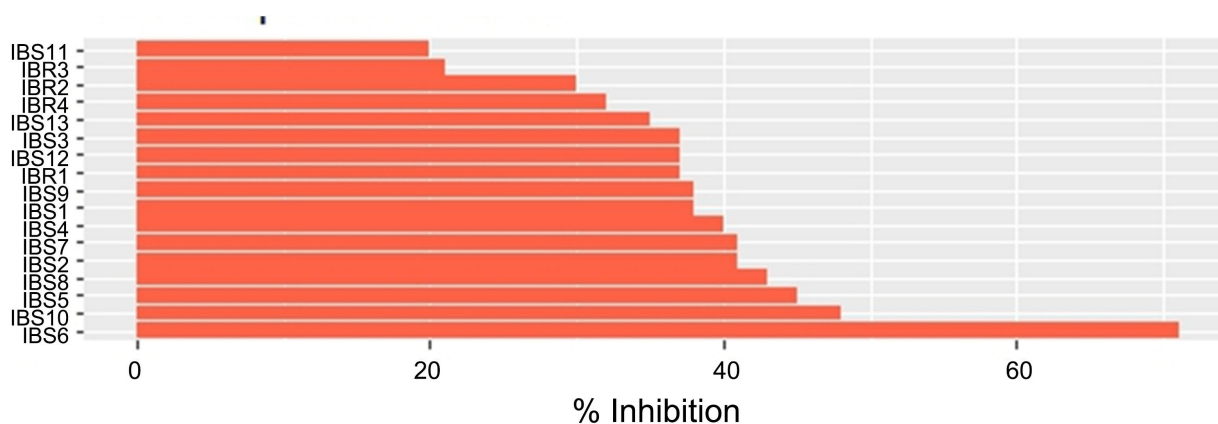


Figure 7. Percentage of inhibition of *Ganoderma sp.* induced by isolated bacteria.

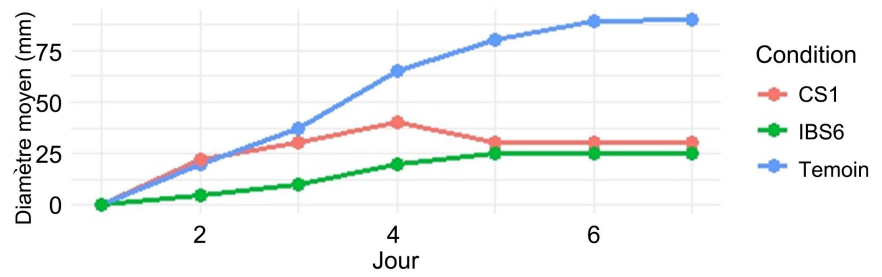


Figure 8. Effect of CS1 and IBS6 on the radical growth of *Ganoderma sp.*



Figure 9. Interaction between *Ganoderma sp.* and CS₁.



Figure 10. Interaction between IBS₆ and *Ganoderma sp.*

3.3. Microorganisms Identification

3.3.1. Fungus (CS1)

1) Macroscopic description

The macroscopic characterization of the fungus (CS1) was conducted on 7-day-old cultures. This fungus has aerial hyphae forming a dense network. The colonies are initially whitish and then turn green after sporulation, which starts on the 4th

day with the first concentric circle. The texture is cottony, and the mycelium is composed of branched filaments. Fungus (CS1) shows fast growth in culture medium and the development of conidia with green-yellow color (**Figure 11**).



Figure 11. Fungus (CS₁).

2) Microscopic description

Under the microscope, the fungus (CS1) has phialides, specialized cells that produce bottle-shaped conidia. The conidia are green and spherical, characteristic of asexual spores borne on branched conidiophores (**Figure 12**).

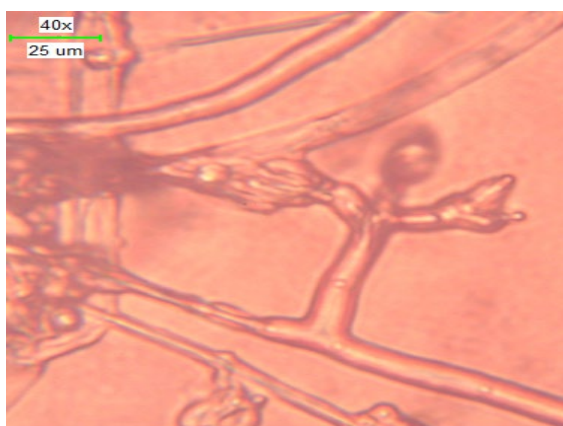


Figure 12. Microscopic structure.

3.3.2. Bacterium (IBS₆)

1) Macroscopic characteristics

After inoculation on PCA medium and incubation for 48 hours at 27°C, the macroscopic characteristic of IBS6 Bacterium are summarized in table below (**Figure 13**).

Code	shape	Color	Elevation	size	Surface	opacity	edge
IBS6	Irregular	yellow	bomb	punctiform	smoth	translucent	serrated



Figure 13. Bacterium (IBS₆).

2) Microscopic characteristics

Gram staining performed on colonies of bacterium (IBS₆) reveals that this bacterium is Gram-positive and appears as violet rods.

a) Biochemical test

The biochemical test revealed that bacterium IBS₆ is catalase-positive and oxidase -negative.

3.3.3. Molecular Identification of Microorganisms (CS1 and IBS6)

The BLAST results correspond to the similarity between the queried sequences and biological sequence in the NCBI database. Two significant matches were obtained: for code IBS₆, a similarity of 99.79% was observed with *Bacillus subtilis* (GenBank accession MT571500.1) and CS1, a perfect match of 100% identity was found with *Trichoderma virens* Genank (accessionMN102106.1).

4. Discussion

4.1. Sampling and Isolation of *Ganoderma sp.*

This study aimed firstly to highlight the microbial biodiversity within the palm grove ecosystem. The results showed a significant qualitative and quantitative diversity in the two studied components, soil and root endophytes. The isolation of potential biological control agents of phytopathogens requires sampling from the rhizosphere of plants to be protected that are growing in soil infested with the target pathogens [19]. Furthermore, it is important to select roots of vigorous and healthy plants located in the area where the disease is present, in order to identify microorganisms that have developed effective defense strategies against phytopathogens.

Isolation from healthy oil palm roots revealed a predominance of fungi (64%) compared to bacteria (36%). These results are similar to those that obtained by [20] who reported a proportion of 66.31% of fungi against 33.04% of bacteria dur-

ing their search for indigenous microorganisms of cacao antagonistic to *Phytophthora palmivora*.

4.2. Isolation and Purification of Microorganisms

In the soil, a high proliferation of the two categories was observed, with bacteria being the dominant group (75%). [21] reported that bacteria are numerically abundant organisms in the soil, especially in rhizosphere. It is the preferential site for indigenous microorganisms with potential antagonistic activity against *Ganoderma sp.* because they occupy the same ecological niche [20] [21].

Secondly this study aimed to identify indigenous microorganisms with antagonistic activity against *Ganoderma sp.* To achieve this study, *in vitro* screening was conducted on microbial isolates to select those showing promising efficacy against this pathogen.

Mycelium isolated from the carpophore of *Ganoderma sp.* exhibited morphological characteristics consistent with those reported for *Ganoderma sp.* by [22] and [23].

4.3. Microorganisms Identification

The result of the *in vitro* dual culture test showed that the bacterium *Bacillus subtilis* (IBS₆) and the fungus (CS₁) were able to inhibit the radial growth of *Ganoderma sp.* with PIRG value of 72% and 66% respectively. The selection of the two microorganisms as biological agents depends largely on their effectiveness *in vitro* result against *Ganoderma sp.* Some studies have also revealed that *Ganoderma sp.* has been inhibited to some extent by some biocontrol agents, namely *Actinomyces*, arbuscular mycorrhizal fungi, *Bacillus spp.*, *Diaporthe spp.*, mycoparasite, *Penicillium*, *Streptomyces spp.*, and *Trichoderma spp.* [24]; [12] tested 3 different species of *Trichoderma* namely *T. asparellum*, *T. virens*, *T. harzianum* reporting that all the *Trichoderma spp.* tested significantly inhibited the growth of *Ganoderma sp.* with PIRG of more than 70% for all the species tested. The inhibition of *Ganoderma sp.* observed in direct confrontations suggests that the significant growth of antagonistic agents allow them to develop more rapidly, colonize the culture medium, and mobilize nutrients to their advantage [25]. This effect could be most likely due to the production of antibiotic, secondary metabolite compounds, or lytic enzymes which could contribute to the direct antagonistic effect degrading *Ganoderma sp.* cell wall and mycoparasitic activities [12] [24]. Moreover, antagonism could be also be caused by production of volatile and non volatile antibiotic acting synergistically with enzymes to counteract the pathogen. The main lytic enzymes produced by the biological control agents are chitinase, glucanase, cellulase and protease. The production of these cell wall degrading enzymes could be responsible for the inhibition of *Ganoderma sp. in vitro*. During direct confrontation between the *Trichoderma virens* (CS₁) and *Ganoderma sp.* significant differences were observed in antagonistic activity. The radical growth diameter of *Ganoderma sp.* control and the confronted were similar until

3rd day, suggesting a delayed antagonistic activity. However after the 3rd day, a significant difference between Control and the treatment diameters was noticed. By the 4th day, *Ganoderma sp.* growth (40 mm) stopped, likely due to space occupation or nutrient depletion. On the 5th day, the diameter of *Ganoderma sp.* reduced to 30 mm, which could correspond to the destruction of a portion of *Ganoderma* mycelium by the antagonistic agent (Figure 10).

For the treatment by *Bacillus subtilis* (IBS₆) the *Ganoderma sp.* growth was slowed from the first day compared to the control, indicating early antagonistic activity. [26] reported that antibiotic production by *Bacillus subtilis* is a process that begins early in the growth of the bacterium, but it is during the stationary phase or in aged cultures that the bacterium releases the largest amount of antibiotic into the culture medium, maximizing its antimicrobial potential. The early antagonistic activity of the bacterium IBS₆ could be attributed to its rapid growth, allowing it to effectively colonize the environment and exert its inhibitory effect [27]. The diameter reached its peak on the 5th day and remained stable until 7th (Figure 10). The lack of physical contact between IBS₆ and *Ganoderma sp.* suggest a potential inhibitory action at a distance, likely due to the production of antibiotics and other secondary metabolites.

5. Conclusion

This study demonstrates that it is possible to isolate indigenous microorganisms from the oil palm ecosystem. Among isolates, a fungus (CS₁) and a bacterium (IBS₆) showed significant antagonistic effects against *Ganoderma sp.* in laboratory conditions. Molecular identification of selected microorganisms revealed that the fungus (CS₁) is affiliated with *Trichoderma virens* and the bacterium IBS₆ with *Bacillus subtilis*. This *in vitro* efficacy can serve as research leads for developing biological control against basal stem rot in oil palms. However, the potential of this approach can only be realized if its efficacy is confirmed through *in vivo* testing and subsequently validated under real-world conditions in oil palms plantations.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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