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Research Article

Bioprospecting Study of Plant Growth Promoting Rhizospheric Bacteria from Oil Palm Plantation as Biological Control Agent of *Ganoderma boninense*

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Abstract

Background and Objective: The prioritisation of oil palm studies involves the exploration of novel bacterial isolates as possible agents for suppressing *Ganoderma boninense*. The objective of this study was to evaluate and characterise the potential of rhizospheric bacteria, obtained from the rhizosphere of oil palm plants, in terms of their ability to demonstrate anti-*Ganoderma* activity. **Materials and Methods:** The study began by employing a dual culture technique to select hostile bacteria. Qualitative detection was performed to assess the antifungal activity, as well as the synthesis of chitinase and glucanase, from certain isolates. The candidate strains were molecularly identified using 16S-rRNA ribosomal primers, specifically the 27F and 1492R primers. **Results:** The findings of the study indicated that the governmental plantation exhibited the highest ratio between diazotroph and indigenous bacterial populations in comparison to the other sites. Out of a pool of ninety bacterial isolates, a subset of twenty-one isolates demonstrated the ability to impede the development of *G. boninense*, as determined using a dual culture experiment. Twenty-one bacterial strains were found to exhibit antifungal activity. Nine possible bacteria were found based on the sequence analysis. These bacteria include *Burkholderia territorii* (RK2, RP2, RP3, RP5), *Burkholderia stagnalis* (RK3), *Burkholderia cenocepacia* (RP1), *Serratia marcescens* (RP13) and *Rhizobium multihospitium* (RU4). **Conclusion:** The findings of the study revealed that a significant proportion of the bacterial population exhibited the ability to perform nitrogen fixation, indole-3-acetic acid (IAA) production and phosphate solubilization. However, it is worth noting that *Rhizobium multihospitium* RU4 did not demonstrate the capacity for phosphate solubilization, while *B. territory* RK2 did not exhibit IAA production.

Key words: 16S rRNA, antagonistic bacteria, *Burkholderia*, diazotrophic, oil palm

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Ganoderma boninense is a fungal parasite that induces physical harm or decay in the basal stem of oil palm trees. The onset of symptoms was observed in the foliage, characterised by the presence of many fronds exhibiting wilting, necrosis and rotted stems in the lower sections. According to the study of Kinge and Mih[1], root rot can result in the inability to effectively absorb water and nutrients from the soil, ultimately leading to malnutrition and dehydration. The incidence of basal stem rot may potentially increase due to unfavourable climatic conditions that are not conducive to the growth of oil palm. Therefore, it is anticipated that the productivity of oil palm in a specific region of Sumatra will become unpredictable and result in unsustainable yields after the year 2050[2].

In contemporary discourse, the prevention of microbial infection caused by *G. boninense* is increasingly recognised as a paramount measure, with disease control assuming a position of utmost importance. The application of physico-chemical and biological treatment methods has been employed to extend the economic viability of oil palm cultivation. The present emphasis on disease prevention management has been centered around the use of biological therapeutic methods, namely the application of antagonistic microorganisms such as bacteria and fungi. These microbes have shown potential antifungal properties against *Ganoderma*, as supported by evidence obtained from *in vitro* and *in vivo* studies. Multiple antagonistic bacteria were subsequently isolated as endophytes in oil palm[3-5] and rhizosphere soil[6]. The most recent bacterial species employed as biocontrol agents for antagonism were documented to be *Trichoderma harzianum*, *T. viride*, *Bacillus* sp., *Pseudomonas fluorescens* and *Gliocladium viride*. These species have demonstrated effectiveness in controlling *G. boninense*[7]. The primary mechanism by which microbial antagonistic activity acts against *G. boninense* is predominantly through antifungal activity, hydrolytic enzyme activity[4] and niche competition[8]. In a systematic manner, it was observed that biocontrol agents were capable of inducing resistance to oil palm through the production of chitinase, glucanase and phenolic compounds as elicitors[9]. Furthermore, it has been shown that many biocontrol agents had the capability to safeguard plants through the synthesis of multiple chemical compounds, which are known as diazotrophic bacteria and have the ability to stimulate plant growth[8].

Diazotrophic bacteria encompass a collection of bacteria, including nitrogen-fixing bacteria and actinobacteria, that exhibit symbiotic or free-living lifestyles. These bacteria possess the capability to convert atmospheric nitrogen into ammonia, hence facilitating the growth of their associated plant hosts[10]. Certain species of diazotrophic bacteria have been observed to possess the ability to impede or hinder the proliferation of fungal pathogens through various mechanisms. These mechanisms include engaging in competitive interactions, producing antifungal compounds, triggering plant systemic resistance, synthesising vitamins and phytohormones, solubilizing inorganic phosphate and suppressing plant ethylene activity. These findings have been documented in scientific literature[8,9,11]. The diazotrophic bacteria that exist in a free-living state can be obtained from the rhizosphere of different crops, including *Burkholderia*, *Azoarcus*, *Bacillus*, *Azotobacter*, *Beijerinckia*, *Pseudomonas*, *Klebsiella*, *Herbaspirillum*, *Dertix*, *Zooglea*, *Arthrobacter* and *Enterobacter*[8,12]. In a study conducted by Madiha *et al.*[13], it was observed *that Burkholderia cepacia*, a type of diazotrophic bacterium, exhibited the ability to inhibit the growth of *Lasiodiplodia theobromae*, a phytopathogenic fungi that affects kenaf (*Hibiscus cannabinus* L.). *Bacillus* species are frequently employed as a biological control agent and biofertilizer[14]. Several organisms of the diazotroph group have been found to possess protective features against *G. boninense* in oil palm, including *Burkholderia*, *Bacillus* and *Pseudomonas*[9,11]. The investigation of antagonistic diazotrophic bacteria as potential options for controlling *Ganoderma boninense* in oil palm is a worthwhile endeavour. The primary objective of this work is to identify novel bacterial strains that possess antagonistic properties capable of inhibiting the growth of *G. boninense*, while also stimulating the growth of oil palm.

MATERIALS AND METHODS

Study area: The research project was conducted from March 2018 to November 2019. The research was carried out in the Physiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Sumatera Utara, Medan, Indonesia.

Sampling sites: Rhizosphere soil around oil palm roots was sampled from three different plantations namely university, government and community. The university plantation was located at Campus of Universitas Sumatera Utara, Medan, Indonesia. Governmental and community-owned oil palm plantations were located at Tanjung Morawa and Bingkat Village respectively, North Sumatra, Indonesia. Rhizosphere soils were collected in composites from 3 sampling points, stored in the sealed plastic bags and preserved at 4°C until

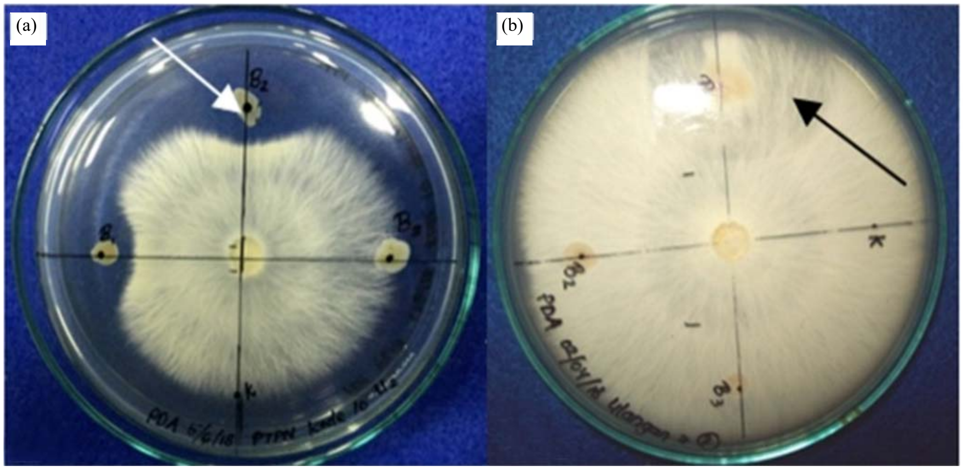

Fig. 1(a-b): Dual culture test of diazotroph bacteria and *Ganoderma boninense* in PDA medium, (a) Clear zone (white arrow) formed in the interaction area between *G. boninense* (center) and bacteria and (b) Less dense hyphal layer of *G. boninense* (black arrow) around of bacterial colony

experimentation. The phytopathogenic fungus, *G. boninense* was obtained from the Indonesian Oil Palm Research Institute (IOPRI), Medan, Indonesia. The fungus was cultivated on potato dextrose agar (PDA Merck®) medium. Diazotrophic bacteria were isolated and cultivated on Ashby's Mannitol Agar (AMA Himedia®).

Isolation of rhizospheric bacteria capable of fixing N: An amount of 10 g rhizospheric soils were suspended with 90 mL distilled water. The soil suspension was shaken for 10 min and then diluted in serial dilution concentrations. An aliquot of 0.1 mL soil suspension from the higher dilution was spread onto nutrient agar (NA) and Ashby's Mannitol Agar (AMA) medium. Bacterial cultures were incubated for 7 day at 28°C. Total colonies of bacterial in each medium were recorded and then the bacterial colonies were sub-cultured to be purified.

Dual culture assay: The thirteen bacterial morphotypes previously grown on Ashby's medium from each study sites were tested for their antagonistic activities against *G. boninense* by dual culture methods on PDA medium. Bacterial cell was inoculated 0.5 cm at distance beside the edge of 3-days old *G. boninense* colony (Fig. 1). Yurnaliza *et al.*[4] studied the colony growth inhibition (CGI (%)) of *G. boninense* by diazotrophic bacteria was calculated as formulation:

$$\text{CGI}(\%) = \frac{\text{R1} - \text{R2}}{\text{R1}} \times 100$$

R1 : Radial growth of *G. boninense* colony without competitor (control)
R2 : Radial growth of *G. boninense* colony with diazotrophic bacteria side

Characterization of isolated bacteria: Macroscopic and microscopic characters of bacterial colonies and cells were observed. Macroscopic characters observed were color, edge, shape, surface and elevation of bacterial colonies while microscopic characters were cell shapes and Gram staining. The biochemical characteristics observed were citrate, catalase activity and motility test.

Antifungal test: Antifungal compounds produced by diazotrophic bacteria were prepared in two fermentation mediums. The selected bacteria were grown on PDB medium for 48 hrs at room temperature. Fermentation medium was extracted using two different solvents (MeOH and EtOAc and concentrated in vacuo. The dry extracts were dissolved in 100% Dimethyl Sulfoxide (DMSO) (w/v) and then tested for its antifungal activity to *G. boninense*. Both MeOH and EtOAc extracts (50 µL) were spotted onto a sterile paper disc (5 mm in diameter) and placed 0.5 cm at a distance beside the edge of 3 day old of *G. boninense* colony. The CGI was measured according to previous method. The DMSO was used as a negative control.

Chitinase assay: Chitinase activity was assayed qualitatively by observing the clear zone around bacterial colony cultivated on colloidal chitin agar. The colloidal chitin agar was composed of (g/L): 0.7 g K_2HPO_4, 0.3 g KH_2PO_4m, 0.5 g $MgSO_4 \cdot 7H_20$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.001 g $ZnSO_4$, 0.001 g $MnCl_2$ and colloidal chitin (0.2 %). The colloidal chitin was extracted and purified from chitinous shrimp shells (sigma) by acid hydrolysis according to the Skujins method. Diazotrophic bacterias were inoculated onto colloidal chitin agar and incubated at room temperature for 24 hrs. Ratio of the diameter of clear zone and bacterial colony was calculated as chitinase activity.

Glucanase assay: Glucanase activity was assayed qualitatively by observing the clear zone around bacterial colony cultivated on glucan agar medium supplemented with 1% (w/v) laminarin. The clear zone was detected clearly when overnight cultivated bacteria were stained using 1% congo red for 5 min and then rinsed with 1 M NaCl[15]. The ratio of the diameter of clear zone and bacterial colony was calculated as glucanase activity.

Phosphate solubilization assay: Phosphate solubilizing activity of diazotroph bacteria was determined by clear zone formation when bacteria cultivated on Pikovskaya agar medium. The clear zone was recorded after 5 day incubation at 28°C. The phosphate solubility index was determined by the comparing the diameter of the clear zone and bacterial colony.

Quantification of indole acetic acid (IAA): Indole acetic acid production was determined based on the colorimetric method. An aliquot of 0.5 mL bacterial suspension (0.5 McFarland standard) was added into 5 mL Ashbys liquid medium containing 0.1% (w/v) L-tryptophan and incubated at 28°C for 48 hrs under agitation at 100 rpm. The bacterial culture was then centrifuged at 5000 rpm for 120 min. The supernatant was added into Salkowski's solution at ratio of 1:4 ratio and incubated in the dark for 30 min. Purple color formation was measured using Shimadzu UV mini-1240 UV-VIS Spectrophotometer Car No. 206-89175-92, Shimadzu, 2008, China at a 535 nm. The concentration of IAA in the samples was estimated by comparing A_{535} of samples with standard curve of IAA.

Quantification of N-fixing capacity: Diazotrophic trait of bacterial isolates was assayed qualitatively by observing the formation of pellicle and quantitatively through Ammonium (NH_4) production in N-free medium. Production of pellicle was observed on semi-solid Ashby's medium. The cultivated medium was incubated at 28°C for 10 day. The pellicle formation in the surface medium was measured[17]. Ammonium production in the N-free medium was observed in Ashby's liquid medium. The cultured bacteria were incubated at 28°C for 48 hrs under agitation at 120 rpm. The cultivated medium was centrifuged at 1,0000×g, at 4°C for10 min. An aliquot of 3 mL supernatant was added into 0.13 mL Nessler solution (Merck®) and then incubated at 28°C for 30 min. The ammonium concentration in medium was estimated by comparing A_{435} of samples with standard curve of ammonium solution (NH_4Cl).

Molecular identification of potential strains: Identification of diazotrophic bacteria was based on 16S rRNA similarity. Polymerase Chain Reaction (PCR) technique along with universal primer bacteria identification, 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3' and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3' was used to amplify gene of interests. Genomic DNA (20 ng) was used as the template and then mixed with an EF-Taq (Sol Gent, Korea) in a 30 µL PCR reaction. The PCR was conditioned as follows: Pre-denaturation (95°C, 2 min), followed by 35 cycles of denaturation (95°C, 1 min), annealing (55°C), elongation (72°C, 1 min) and final extension (72°C, 10 min). A multiscreen filter plate (Millipore Corp., Bedford, Massachusetts, USA) was used for purification of amplicons. The sequencing reaction was performed using PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit. All PCR and sequencing processes were conducted by Macrogen Inc., in South Korea. DNA sequence results were edited using the Bioedit program and stored in the FASTA file and then searched for similarities with the database in the EZ Taxon site. A total of 10-15 DNA sequence references producing high similarities were chosen to be analyzed for their genetic distance relationships and then aligned using MUSCLE in MEGA6. Phylogenetic tree was constructed using neighbor-Joining method in MEGA 6 with 1000× bootstrapping.

RESULTS

Population of rhizospheric and diazotrophic bacteria: The overall bacterial community, consisting of cosmopolitan and diazotrophic species, exhibited variation across three distinct locations inside the rhizosphere of oil palm plants. In general, the nutrient agar (NA) medium exhibited a greater capacity for bacterial colony recovery compared to the N-free medium. The rhizobacterial population was found to be more abundant on Campus USU when considering different sites. In contrast, the diazotrophic rhizobacteria ratio was found to be higher in the governmental plantation, while the smallest ratio was observed on Campus USU, as indicated in Table 1.

Table 1: Total bacterial population of oil palm rhizobacteria obtained from three sites based on isolation medium

	Total bacteria population (CFU/mL)		
Rhizosphere oil palm location	General medium (nutrient agar)[a]	N-free medium (diazotrophic)[b]	Ratio$^{b/a}$ (%)
Campus USU	1.44×10^5	5.5×10^4	38
Governmental plantation	1.17×10^5	9.0×10^4	76
Community plantation	1.05×10^5	6.3×10^4	60

Table 2: Selective test for dual test result, anti-*Ganoderma* activity and plant growth promoting mechanism support of diazotrophic bacteria from rhizosphere soil

		Anti-*Ganoderma* activity/CGI (%)			
Isolate code	CGI (%)	MeOH	EtOAc	Chitinolytic index	Glucanolytic index
RU01	44.40	38.09	27.27	0.00	0.40
RU02	12.20	4.70	22.22	0.00	0.00
RU03	37.70	19.04	11.11	0.00	0.88
RU04	66.60	13.63	6.25	0.00	0.00
RK01	30.00	10.52	6.60	0.00	0.60
RK02	47.30	21.05	20.00	0.00	0.66
RK03	36.80	5.20	26.60	1.66	0.00
RK04	18.75	4.54	20.00	0.00	0.20
RP01	33.30	10.52	26.6	0.00	1.14
RP02	31.40	31.57	6.60	0.00	0.57
RP03	34.20	10.52	13.33	0.00	1.12
RP04	18.75	4.70	31.57	0.00	0.55
RP05	25.00	28.57	10.52	0.00	0.44
RP06	14.80	19.04	21.05	0.00	0.55
RP07	11.10	16.60	12.50	0.00	0.20
RP08	12.20	29.16	12.50	0.00	0.88
RP09	8.00	25.00	20.83	0.00	0.50
RP10	20.50	5.00	12.50	0.00	0.28
RP11	10.34	25.00	18.75	0.00	0.63
RP12	18.80	30.00	18.75	5.33	0.87
RP13	45.20	27.27	31.25	2.80	0.00

Antagonistic properties of isolated bacteria against *Ganoderma boninense*: A total of ninety isolates of diazotrophic bacteria were subjected to evaluation for their antifungal activity using the dual culture method. The findings revealed that out of the examined isolates, twenty-one exhibited antagonistic properties against *G. boninense*. The range of colony growth inhibition (CGI) percentages observed in this study varied from 8 to 66.6% (Table 2). The highest percentage of computer-generated imagery (CGI), specifically 66.6%, was detected in RU04, which corresponds to the USU campus. During the dual culture test, it is possible for antagonistic bacteria to synthesize antifungal chemicals, as evidenced by the observation of a clear zone in the interaction region between the bacterium and fungus. The hyphae of *Ganoderma boninense* exhibited a directional growth pattern, away from the bacterial colonies, as depicted in Fig. 1a. Nevertheless, the visual clarity of the inhibitory zone may vary. Furthermore, it is noteworthy that there is a discernible occurrence of a hyphal layer with reduced density in the vicinity of the contact zone, hence indicating a potential presence of antifungal activity (Fig. 1b).

Antifungal activity of isolated strains against *Ganoderma boninense*: The fermentation broth of diazotrophic bacteria exhibited antifungal properties against *G. boninense*, as observed in the MeOH and EtOAc extracts. The percentage of computer-generated imagery (CGI) ranged from 4.54-38.09%. The results obtained from the study indicate that a significant proportion of the bacterial isolates exhibited substantial antifungal properties upon extraction using methanol as the solvent, as shown in Table 2. The emergence of mycelium extending beyond the vicinity of antifungal extracts is evident in the observation depicted in Fig. 2a. The MeOH extract had the most potent antifungal activity, with RU01 extract demonstrating the maximum efficacy at 38.09% and it is evident that the hyphae layer of *G. boninense* has a lower density (shown by the black arrow) around the bacterial colony (Fig. 2b). Similarly, the EtOAc extract from RP04 extract displayed notable antifungal activity, with a percentage of 31.57% (Table 2).

Enzymatic properties of isolated strains: Chitinolytic activity was only observed from 3 isolates out of 21 isolates tested, namely RP12, RP13 and RK03. The chitinolytic activities of

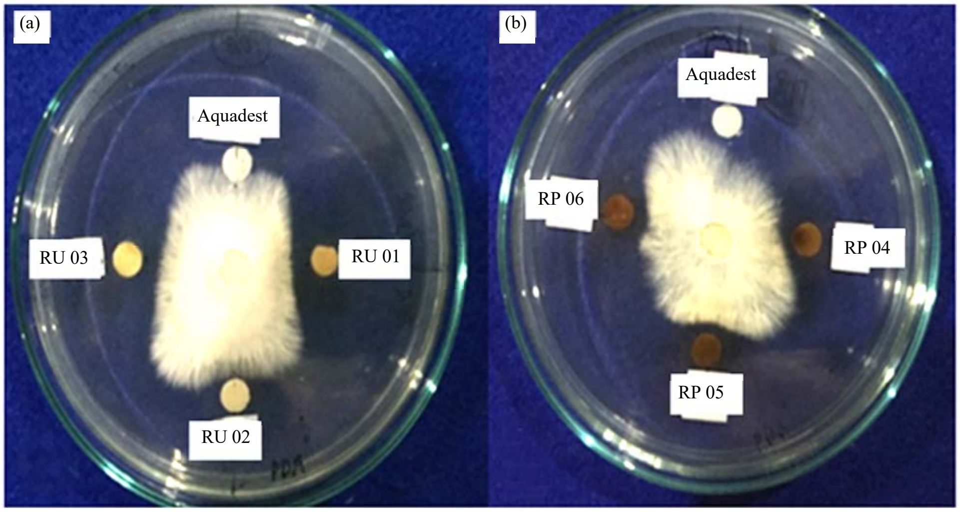

Fig. 2(a-b): Anti-*Ganoderma* activity of diazotrophic bacterial extract from several isolates diazotrophic bacteria against *Ganoderma boninense*, (a) Methanol extract and (b) Ethyl acetate extract

Table 3: Colony morphological properties of diazotrophic bacterial isolates are distinct from those of the resultant isolates

		Colony morphology						
Code	Gram	Shape	Elevation	Margin	Color	Motility	Catalase activity	Citrate utilization
RU01	-	Irregular	Raised	Entire	Brownish yellow	+	+	-
RU02	+	Irregular	Raised	Undulate	Orange	-	-	+
RU03	-	Irregular	Raised	Curled	Brownish yellow	+	-	+
RU04	-	Irregular	Raised	Entire	White	+	+	-
RK01	-	Irregular	Raised	Undulate	Yellow	+	-	+
RK02	-	Irregular	Raised	Curled	brownish yellow	+	+	-
RK03	-	Circular	Flat	Entire	White	+	+	-
RK04	-	Irregular	Flat	Undulate	White	-	-	+
RP01	-	Irregular	Flat	Undulate	Brown	+	+	-
RP02	-	Irregular	Flat	Curled	Yellow	+	+	-
RP03	-	Circular	Raised	Entire	Yellow	+	+	-
RP04	-	Irregular	Raised	Undulate	White	+	-	+
RP05	-	Irregular	Raised	Curled	Brownish yellow	+	+	-
RP06	+	Circular	Raised	Entire	White	+	-	+
RP07	+	Circular	Raised	Entire	White	+	-	+
RP08	-	Circular	Raised	Entire	White	+	-	+
RP09	-	Irregular	Raised	Curled	White	-	-	+
RP10	-	Circular	Raised	Entire	White	-	-	+
RP11	-	Circular	Raised	Entire	White	+	-	+
RP12	-	Circular	Flat	Entire	Yellow	+	+	-
RP13	-	Irregular	Flat	Lobate	Red	+	+	+

bacterial isolates were evidenced by the formation of clear zones around colonies. The clear zone size indicated the chitinolytic activity to hydrolyze polymeric chitin into monomeric compound, N-acetyl glucosamine. The chitinolytic index of 3 diazotrophic bacteria ranged between 3.6-6.3 (Table 2). Glucanolytic activity was observed in 17 of 21 isolates tested. Glucanolytic index ranged between 0.2-1.14. Laminarin extracted from *Laminaria digitata* in a glucan medium was added as a carbon source. The formation of a clear zone around bacterial colonies indicated the glucanolytic activity of laminarin being hydrolyzed into oligomer or monomer by glucanase. The glucanase acts by breaking the β-1,3 and β-1,6-bond of glucan.

Species identification result of isolated strains: Table 3 presented a summary of the morphological traits exhibited by 21 isolates of diazotrophic bacteria. The observed colony morphologies exhibit irregular and circular shapes, characterised by edges that are either whole, undulated, or curled. The topography of the colonies was primarily

Table 4: Molecular identification results based on 16SrRNA gene of anti-*Ganoderma* selected diazotrophic bacteria and the similarity with related bacteria in EZ-Taxon database

Isolate	Source of isolates	Identification based on EZTaxon databases	Similarity (%)	Accession code
RK 2	Community	*Burkholderia territorii* LMG 28158	99.79	LK023503
RK 3	Community	*Burkholderia stagnalis* LMG 28156	99.86	LK023502
RP 1	Governmental	*Burkholderia cenocepacia* LMG 16656	99.86	JTDP01000003
RP 2	Governmental	*Burkholderia territorii* LMG 28158	99.86	LK023503
RP 3	Governmental	*Burkholderia territorii* LMG 28158	99.93	LK023503
RP 5	Governmental	*Burkholderia territorii* LMG 28158	99.86	LK023503
RP 13	Governmental	*Serratia marcescens* subsp. *marcescens* ATCC 13880	99.59	JMPQ01000005
RU 1	Campus USU	*Burkholderia territorii* LMG 28158	99.79	LK023503
RU 4	Campus USU	*Rhizobium multihospitium* HAMBI 2975	100.0	jgi.1052913

Table 5: Plant growth promoting trait from potential of anti-*Ganoderma boninense* of diazotrophic bacteria

Bacterial isolates	Ammonium concentration (ppm)	IAA concentration (ppm)	P-solubilization index
Rhizobium multihospitium RU04	1.16	1.22	0.00
Burkholderia territorii RK02	2.89	0.00	0.83
Serratia marcescens RP13	1.39	10.24	2.44
Burkholderia territorii RU01	3.21	0.13	6.54

characterised by flat terrain, while the coloration exhibited a range from cream to crimson. The majority of isolates exhibited Gram-negative characteristics, demonstrated the ability to use citrate, displayed motility and produced catalase, as indicated in Table 3. The primary objective of this study was to perform molecular identification of diazotrophic isolates, with a specific focus on nine possible anti-*Ganoderma* isolates. Table 4 presented the summarised outcomes of the molecular identification process conducted on the possible diazotrophic isolates. The 27F-1492R primer, which is widely recognised as a universal tool for bacterial identification, was employed in this analysis. The process of identification yielded three genera, namely *Burkholderia*, *Serratia* and *Rhizobium*. The similarity levels of nine isolates, which were compared to the database in the EZ Taxon genbank database anged from 99.59-100%.

Plant growth promoting properties of isolated strains: Several plant growth boosting features were evaluated in diazotrophic isolates, including nitrogen fixation, indole-3-acetic acid (IAA) synthesis and P-solubilization, as presented in Table 5. All isolates of *Ganoderma* were shown to possess the ability to fix nitrogen, as evidenced by their growth in a nitrogen-free media. The N-free medium is a widely used medium that has been specifically designed to preferentially separate non-symbiotic nitrogen fixing bacteria. Therefore, the measurement of nitrogen fixation was conducted using the Nessler method to quantify ammonium formation in Ashby's liquid medium devoid of nitrogen. The findings of the study revealed that RU01 exhibited the highest concentration of ammonium, suggesting its potential as a nitrogen-fixing isolate.

Quantitative assay of IAA production was only screened on 4 selected bacteria. Only three isolates (RU04, RP 13 and RU 01) were able to produce IAA, while RK02 has no activity. The IAA production from three isolates was varied from 0.13-10.24 ppm. The presence of IAA was observed through the color change of medium to pink when added with Salkowski reagent. The pink color intensity correlates with higher IAA concentration. The IAA concentration was influenced by L-tryptophan as a precursor. The absence of L-tryptophan will yield a minute concentration of IAA in the medium. In this research, IAA production was recorded in the presence of L-tryptophan in the fermentation medium. *Serratia marcescens* RP 13 produced the highest IAA concentration among others. The IAA concentration produced by two strains of *Burkholderia territorii* (RK 02 and RK 01) was only detected in low concentration.

Although, IAA is constitutively produced by the plant, contribution of exogenous IAA by bacteria is beneficial in promoting plant growth. Phosphate solubilizing activity was tested on 4 selected isolates, assayed on the Pikovskaya medium supplemented with insoluble Tricalcium Phosphate ($Ca_3(PO_4)_2$). The clear zone formed around bacterial colonies indicates a positive result of phosphate solubilization. Insoluble form of tricalcium phosphate has been converted into soluble form $PO4^{3+}$. The highest P-solubilization index was observed from *Burkholderia territorii* RU01 (6.54), followed by *S. marcescens* RP 13 while *Rhizobium multihospitium* RU04 produced no activity (Table 4). Phosphate solubilizing activity of *B. territorii* RU01 was better than *B. territorii* RK02. The present study employed neighbor-joining methods, specifically the Kimura 2-parameter model and bootstrap phylogeny test, to build a phylogenetic reconstruction of

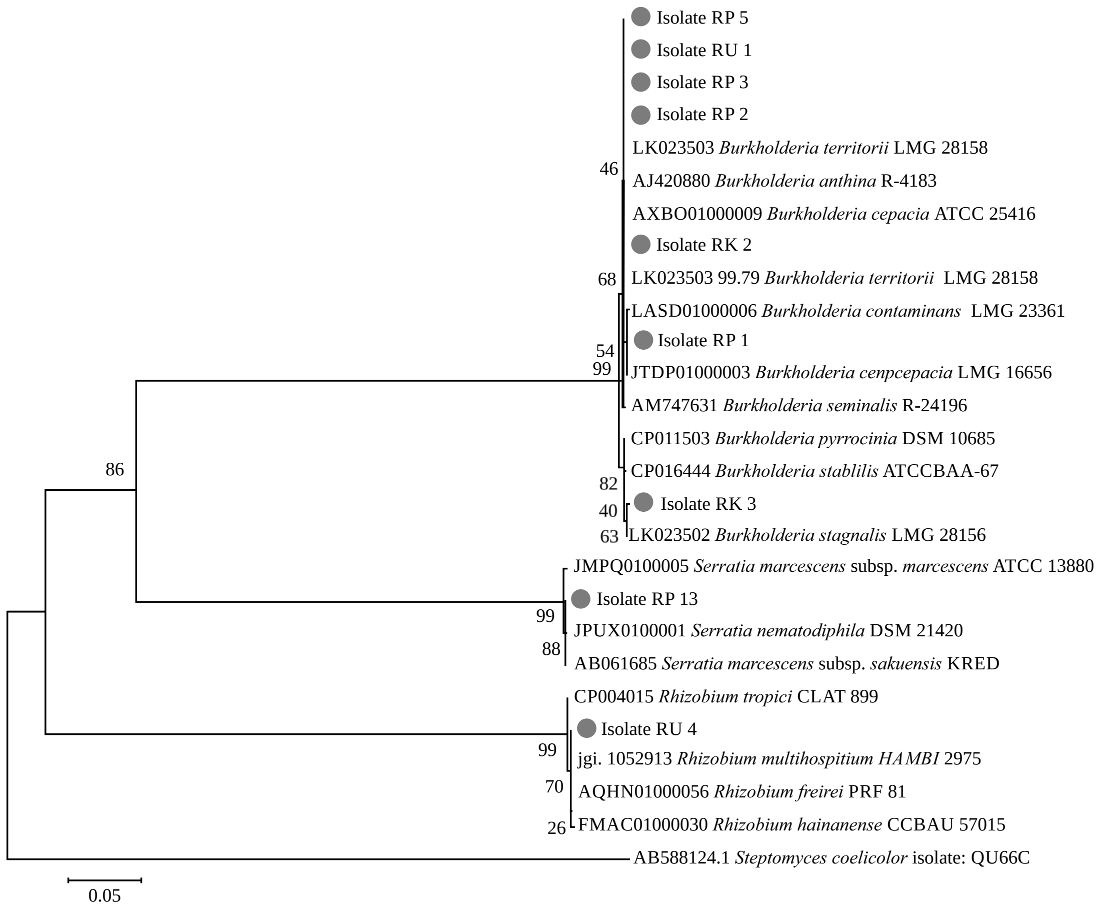

Fig. 3: The phylogenetic analysis of 9 potential bacterial candidates resulted in the identification of 5 distinct species: *Burkholderia territorii* (RK2, RP2, RP3 and RP5), *B. stagnalis* (RK3), *B. cenocepacia* (RP1), *Serratia marcescens* (RP13) and *Rhizobium multihospitium* (RU4), Using MEGA6, the analysis is conducted at the molecular level

The bar represents a 5% estimated phylogenetic divergence

diazotrophic bacteria from oil palm rhizosphere soil and related bacteria. This reconstruction was conducted for 100 replicates. The phylogenetic tree is accurately depicted, with branch lengths expressed in units that correspond to the evolutionary distances employed for inferring the tree. The evolutionary analysis was performed using the MEGA6 software (Fig. 3).

DISCUSSION

The abundance of rhizospheric and diazotrophic bacteria within an oil palm plantation, as observed in this study, is significantly impacted by various factors, including the geographical placement of the oil palm and the management practices employed within the plantation. The primary emphasis of professional management will be directed towards the promotion and maintenance of optimal plant and soil health. The high prevalence of diazotrophic bacteria in the governmental plantation may be attributed to the diligent cultivation practices and effective crop management implemented by the responsible party. The provision of inorganic fertilisers is expected to facilitate the development of plants and induce changes in the composition of soil microorganisms inside the rhizospheric area. Prior research has documented various parameters that could influence the population of microorganisms in the rhizosphere. These factors include plant species, soil location organic and soil management practices, salt levels, nitrogen fertilisation and ammonium concentration in the soil[16-21]. The diversity of rhizospheric microorganisms has been found to be influenced by various factors, including the ontogenic stage of the plant, its health status, the type of soil, the treatment of soil and litter, as well as environmental factors in the vicinity of the roots and interactions among microorganisms in the root region[22,23]. The utilisation of nitrogen fertiliser during the initial phases of plant development has been observed to have a negative impact on the population of diazotrophic bacteria in the rhizosphere[22]. This study posits that the observed differences in diazotrophic bacteria found in the rhizosphere of oil palm trees can be attributed to the application of fertiliser. The implementation of an integrated management system for oil palm plantations, jointly controlled by the government and local populations, has proven effective in enhancing the diversity and abundance of diazotrophic bacterial communities inside the rhizosphere.

The evaluation of antagonistic bacterial isolates as a potential biocontrol agent was conducted by a dual culture test. The conducted comparable methodologies to identify antagonistic diazotrophic bacteria from rice endophytes that could effectively combat various soil-borne fungi[23]. The observed reduction in hyphal density in this study is likely attributable to the activity of hydrolytic enzymes, such as chitinase or glucanase, produced by bacteria that triggered lysis of the *G. boninense* cell wall. Chitin and glucan are crucial constituents within the cellular walls of fungi. In the dual culture test, it was shown that the mycelial growth of *G. boninense* exhibited anomalies, suggesting a hindered or stunted growth. Previous research conducted by Yurnaliza *et al.*[4] has shown the presence of certain irregularities, such as early branching and crookedness in *G. boninense* mycelium. These irregularities are believed to be a consequence of the antifungal properties exhibited by endophytic fungi. The primary mechanisms or targets of antifungal drugs predominantly involve the interference with cell wall formation, change of membrane permeability, disruption of nucleic acid and cellular proteins and modulation of cellular physiology, including the suppression of signal transduction pathways and microtubule aggregation[24].

The present study aimed to evaluate the yet unknown mechanism underlying the antifungal activity exerted by diazotrophic bacteria against *G. boninense*. In relation to the characteristics that promote plant growth, it was observed that all isolates exhibited the ability to perform nitrogen fixation, however only a limited number of isolates shown the capacity to create indole-3-acetic acid (IAA) and solubilize phosphate[25]. Diazotrophic bacteria are sometimes referred to as nitrogen-fixing microorganisms; however, it should be noted that not all of them possess the capability to solubilize phosphate or create indole-3-acetic acid (IAA). The phosphate solubilizing activity exhibited by the diazotrophic isolates was attributed to the production of phosphatase enzymes and organic acid compounds[25]. The production of organic acid by bacteria has been found to be highly efficacious in modifying the solubility of phosphate. The utilisation of phosphate solubilizing microorganisms is a significant approach in enhancing the release of bound phosphorus and improving the accessibility of mineral phosphorus in agricultural soil[26].

Other traits of biofertilizer agents were the ability to produce fungal inhibitor. The antifungal activity of diazotrophic bacterial species has been reported such as *Burkholderia*, *Bacillus*, *Pseudomonas* and *Serratia* are reported to be effective in inhibiting fungal pathogens[27-30]. Mechanism of antifungal activity by diazotrophic bacteria may be a contribution of interacting activity, i.e. chemical compound, hydrolytic enzyme or volatile organic compound. *Bacillus* sp. produced iturines and bacillomycins compounds which are known as strong antifungal compounds and also many other antimicrobial peptides[31]. Burkholdines produced by *Burkholderia ambifaria* is a cyclic lipopeptide which acts as a potential antifungal compound[31]. *Burkholderia* spp. also produced the pyrrolnitrin (prn) antibiotic, a common broad-spectrum antibiotic which was also produced by several *Pseudomonas* and *Serratia*[32].

Chitinolytic activity of diazotrophic bacteria was confirmed through formation of clear zone around colonies grown in colloidal chitin medium which indicated a structural change of chitin into soluble derivatives. Chitinase enzyme hydrolyzes the polymeric chitin into N-acetyl glucosamine monomer. The consequence of chitin structural change is the increasing pH in the clear zone. Adding color pH indicator (Bromocresol purple) into medium was indicate chitin hydrolysis by the color changes from purple to yellow. The purple color of Bromocresol purple indicate an alkaline environment around colonies. Chitinolytic activity of some diazotrophic bacteria has been reported by some researchers[32,33]. They reported that the chitinase enzyme of *Serratia marcescens* has antifungal activity against several pathogenic fungi, namely *Rhizoctonia solani*, *Bipolaris* sp., *Alternaria raphani* and *Alternaria brassicicola*. Chitinase enzyme was also produced by *Bacillus* and *Pseudomonas*[32,33]. Most bacterial chitinase is included in family 18 of the glycosyl hydrolase except chitinase from *Burkholderia*[34].

In contrary, glucanase activity was displayed by almost all diazotrophic isolates. Structurally, chitin and glucan are cell wall-forming polymers in fungi. In ascomycete and basidiomycete, the surface structure of hyphae and in sclerotia are rich in glucans. The ability of bacteria to hydrolyze chitin and glucan was also closely related to its ability as an antifungal. The endo β 1-3 glucanase enzymes produced by

Paenibacillus disrupted the structure of the phytopathogenic fungal cell walls of *Pythium aphanidermatum*, *Rhizoctonia solani* AG-4[35] and *Pseudomonas* sp. The EA6 produced β-1,3 and β-1,4 glucanase enzymes which showed strong inhibitory activity which act by hydrolyzing the fungal cell wall of *Phytophthora parasitica*[36].

In addition, diazotrophic bacteria exhibit antifungal activity by producing protease and lipase, siderophore, ammonia and cyanide (HCN)[37]. In their study, the antifungal properties exhibited by a total of 1219 bacterial isolates[37]. Their findings revealed that less than half of these isolates (i.e., less than 50%) had the ability to create antifungal compounds, while a mere 11% exhibited the production of chitinolytic enzymes. The enhancement of antifungal action of antifungal drugs is reliant on the presence of chitinolytic and glucanolytic enzymes[36,37]. This study encompasses six species from the *Burkholderia cepacia* complex (BCC), namely *B. territorii* (RK2, RP2, RP3, RP5, RU1), *B. stagnalis* RK3 and *B. cenocepacia* RP1. The members of the BCC (*Burkholderia cepacia complex*) are a subgroup of β-proteobacteria, which are classified as Gram-negative bacteria. They have a broad distribution in various environments, including the rhizosphere, plants (as endophytes), animals and even as human pathogens[38]. *Burkholderia cepacia* demonstrated the ability to inhibit various phytopathogenic fungi, including *Rhizoctonia solani*, *Monochaetia hirta*, Fusarium monilia, *F. semitectum*, *Curvularia lunata*, *F. Solani*, *F. graminearum*, *Alternaria alternata*, *F. oxysporum* f. sp. *spinaciae*, *Bipolaris sorokiniana*, *Colletotrichum lindemuthianum* and *Candida albicans*[27]. In a separate investigation, it was observed that *Burkholderia gladioli* pv. *agaricicola* (Bga) exhibited volatile antifungal properties against various phytopathogenic fungi, including *Botrytis cinerea*, *Aspergillus flavus*, *Penicillium digitatum*, *Penicillium expansum*, *Aspergillus niger*, *Phytophthora cactorum* and *Sclerotinia sclerotiorum*. The antifungal properties of *Burkholderia* are not just attributed to its antifungal chemicals but also to its hydrolytic enzyme activities, such as chitinase, glucanase and protease[27].

Interesting finding in our study was the antifungal activity of *Rhizobium multihospitium* RU4. The antagonistic activity of this strain against *G. boninense* was the first reported in this research. *Rhizobium multihospitium* RU4 produced the highest CGI percentage against *G. boninense* with an antifungal mechanism. However, *Rhizobium multihospitium* RU 4 was not able to produce chitinase and glucanase. The ability of *Rhizobium* as biocontrol agent to phytopathogenic fungi has been summarized[39]. Biocontrol activities of *Rhizobium* involved several mechanisms i.e., antibiotics, antifungal metabolites, Hydrogen Cyanide (HCN), mycoparasitism, siderophore, competition and induced plant resistance and growth-promoting activity. As a diazotrophic bacteria, *Rhizobium* was reported to produce IAA exogenously and to solubilize insoluble phosphate[39]. *Rhizobium* also produced exopolysaccharide (EPS) which stimulated plant growth in arid soil[40].

Serratia marcescens sub sp. *marcescens* RP 13 is other diazotrophic isolate from the oil palm rhizosphere which demonstrated potential antagonistic activity against *G. boninense*. Mechanism of inhibition of this bacterium was by synthesis of antifungal compounds and chitinase. *Serratia marcescens* is a rod-shaped gram-negative bacterium from Enterobacteriaceae, also known as opportunistic pathogen in crops and in human infection with wide distribution in environment along with ecological function either as mutualistic or pathogenic to other organisms. Ecological habitat of *S. marcescens* may vary from rhizosphere, endophytes, dirt until sterile sites when infected the human environment[40]. *Serratia marcescens* was also reported as potential PGPR and biocontrol agent against phytopathogenic fungi[41]. The mechanism of *S. marcescens* to lyse fungal hyphae of Zoomycete and Basidiomycete[42]. *Serratia marcescens* was reported to attach and move along hyphal growth and then killing the fungi with or without chitinase[42].

This research has useful implications for oil palms, since it helps to decrease the presence of *Ganoderma boninense* in the soil and safeguards plants against root rot. The application can be formulated into organic or biological fertilisers. Conducting an *in vivo* study is advised to execute this use on a large scale in a nursery setting. This research is limited by the absence of molecular marker data indicating the presence of a gene that enables nitrogen fixation in this strain.

CONCLUSION

Out of the 90 selected isolates, a total of twenty isolates of diazotrophic bacteria exhibited the capability to suppress the growth of *G. boninense*, as determined using a dual culture experiment. The antagonistic mechanism exhibited by diazotrophic bacteria can be attributed to their antifungal properties, as well as their production of chitinase and glucanase enzymes. There are four diazotrophic bacteria that possess plant growth-promoting characteristics and exhibit potential as biofertilizers. The analysis of 9 candidate bacteria yielded 5 distinct species: *Burkholderia territorii* (RK2, RP2, RP3, RP5), *B. stagnalis* (RK3), *B. cenocepacia* (RP1), *Serratia marcescens* (RP13) and *Rhizobium multisporium* (RU4).

SIGNIFICANCE STATEMENT

This research found that the antagonistic mechanism exhibited by diazotrophic bacteria can be attributed to their antifungal properties, as well as their production of chitinase and glucanase enzymes. This research will help researchers reveal there are four diazotrophic bacteria that possess plant growth-promoting characteristics and exhibit potential as biofertilizers. Thus a new theory emerges about the Bioprospecting of plant growth promoting rhizospheric bacteria from oil palm plantations as a biological control agent of *Ganoderma boninense* that can be achieved.

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