
Efficiency of *Bacillus subtilis* in inhibiting the mycelial growth of *Ganoderma boninense* the causal agent of basal stem rot disease in oil palm (*Elaeis guineensis* Jacq.)

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Abstract This research evaluated the efficacy of biological products derived from *Bacillus subtilis* subsp. *subtilis* RUTs001, *Bacillus subtilis* RUTs002 and *Bacillus subtilis* RUTs003 in inhibiting the mycelial growth of *Ganoderma boninense*, the causal agent of basal stem rot disease in oil palm (*Elaeis guineensis*) on agar media and on mushroom bag. The results demonstrated that the combinations of *B. subtilis* subsp. *subtilis* RUTs001 + *B. subtilis* RUTs003 and *B. subtilis* subsp. *subtilis* RUTs001 + *B. subtilis* RUTs002 emerged as the most effective, inhibiting the mycelial growth of *G. boninense* and resulting in a notable slowdown of mycelial growth. The time required for full colonization of the substrate was recorded at 35 days after inoculation. Followed by commercial *B. subtilis* product achieved full colonization at 34 days. Moreover, the use of bioproduct of *B. subtilis* can degrade mycelium completely within 150 days of its colonization. While in the control, mycelium growth was slower, and full colonized was achieved at 32 days.

Keywords: Biocontrol, Fermentation, Mycelium growth, Substrates

Introduction

Oil palm (*Elaeis guineensis* Jacq.) constitutes a vital economic resource in Thailand, where it underpins a significant portion of the agricultural sector. Thailand ranks as the third-largest producer of palm oil globally, following Malaysia and Indonesia, a position substantiated by extensive documentation in the scientific literature (Arsyad *et al.*, 2020; Saeyang and Nissapa, 2021; Shigetomi *et al.*, 2020). From 2009 to the present day, the area dedicated to oil palm cultivation in Thailand has shown a consistent and notable increase, reflecting the crop's growing importance. In 2024, the production volume of oil palm in Thailand was estimated to have reached approximately 19.06 million tons, underscoring its critical role in the national economy (Walderich, 2024).

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The majority of oil palm plantations and associated refineries are situated in the southern regions of Thailand, where the geographic and climatic conditions are particularly conducive to the successful cultivation of this crop (Kumar and Krishna, 2023; Walderich, 2024). However, the sustainability of this industry is increasingly threatened by basal stem rot (BSR) disease, a devastating affliction primarily attributed to species within the *Ganoderma* genus, with *Ganoderma boninense*, *G. zonatum*, and *G. miniatocinctum* identified as the principal causal agents (Nagappan, 2023; Rakib *et al.*, 2017). This disease has the capacity to infect oil palms at any stage of their growth cycle, with its prevalence often exacerbated by inadequate management practices, leading to incomplete stands of trees and a gradual decline in plantation health, ultimately resulting in tree mortality.

The effect of BSR disease is observed across both young oil palms and mature, fruit-bearing trees, with the latter experiencing more severe consequences (Khoo and Chong, 2023; Zakaria, 2023). In its initial stages the infection may remain symptomless allowing *G. boninense* to colonize the stem and root system undetected. As the disease progresses fruiting bodies emerge serving as a clear indicator of advanced infection (Bharudin *et al.*, 2022). The earliest visible symptoms include the collapse and drooping of lower fronds around the trunk, accompanied by an abnormally high number of unopened spear leaves. Internal examination often reveals significant damage to the stem, with up to 50% of its structure compromised, alongside pronounced basal rotting (Haw *et al.*, 2023). As the disease advances to a severe state, the lower fronds gradually dry out and die, with the damage progressively extending upward toward the crown of the tree. At this critical juncture, all available treatments prove ineffective, as the infection has spread throughout the entire plant, rendering recovery impossible (Bivi *et al.*, 2016). Generally, oil palm trees affected by BSR succumb within two to three years following the appearance of initial symptoms, although some individuals may persist for a slightly longer period. Among the *Ganoderma* species, *G. boninense* is widely regarded as the primary species responsible for the decline of oil palm trees due to BSR, producing characteristic fruiting bodies that are reddish-brown in color with a white margin. These fruiting bodies feature a smooth, glossy, lacquer-coated upper surface, while the lower surface is opaque white and densely covered with small pores that serve as sites for the production of fine brown spores. Internally, the pathogen induces the formation of brown lesions with irregular dark brown margins within the stem. Concurrently, the roots become brittle and prone to breakage, with their internal tissues deteriorating into a friable, powdery texture.

The most effective approach to controlling *G. boninense* involves the integration of cultural practices, biological control measures, and the deployment

of oil palm varieties resistant to BSR (Zakaria, 2023). Among these strategies, the use of antagonistic microorganisms, such as species within the genus *Bacillus*, has demonstrated considerable efficacy in suppressing the growth and infection of *G. boninense* in both nursery settings and field conditions (Alexander and Chong, 2014; Puspita *et al.*, 2020; Rashad and Moussa, 2020).

Therefore, this research study focused on the evaluation of three specific *B. subtilis* strains namely, *B. subtilis* subsp. *subtilis* RUTs001, *B. subtilis* RUTs002, and *B. subtilis* RUTs003 were formulated into biological products and assessed for their ability to inhibit the mycelial growth of *G. boninense* under controlled laboratory conditions and within a mushroom bag system designed to mimic practical agricultural scenarios.

Materials and methods

Collection and isolation of Ganoderma spp.

Basidiocarps of *Ganoderma* spp. were systematically collected from areas exhibiting severe incidence of basal stem rot disease (Figure 1), located in Chian Yai District, Nakhon Si Thammarat Province, Thailand (latitude 8°6" N, longitude 100°4" E), during September 2024. The fruiting bodies were cleaned to surface sterilization using 75% ethanol. Subsequently a small section of inner tissue, measuring 5 × 5 mm, was carefully excised from each sterile basidiocarp using a sterilized scalpel. These tissue samples were then placed onto potato dextrose agar (PDA) supplemented with streptomycin sulfate. The cultures were incubated at a temperature range of 28–32°C for 48 hours, after which hyphal tips were subcultured onto new PDA plates. Preliminary identification of the *Ganoderma* species was conducted based on morphological characteristics observed under a microscope, with definitive confirmation achieved through molecular analysis involving sequencing of the internal transcribed spacer (ITS) region of ribosomal DNA. This molecular identification was performed at the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand.

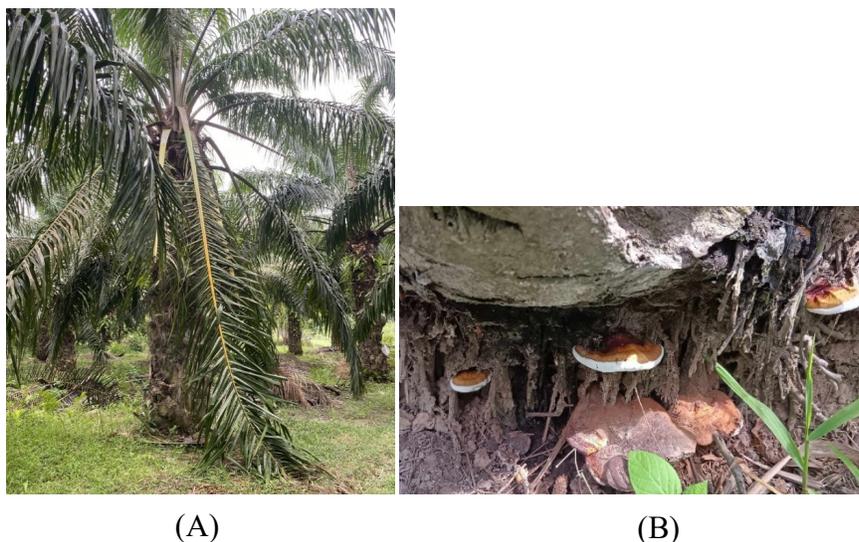


Figure 1. The symptoms of basal stem rot disease of oil palm caused by *G. boninense* (A), the fruiting bodies (B)

Preparation of Bacillus spp. bioproducts

The biological products used in this study were prepared in the laboratory using *B. subtilis* subsp. *subtilis* RUTs001, *B. subtilis* RUTs002, and *B. subtilis* RUTs003. These strains had been previously identified based on the partial sequencing of their 16S rDNA (Zulfikar *et al.*, 2018). The formulations were developed as both individual strain preparations and combinations, with the latter mixed at a 1:1 w/w. Each formulation was standardized to achieve a concentration of approximately 1×10^9 CFU/g WP.

Efficacy of Bacillus spp. in inhibiting mycelial growth of G. boninense

The efficacy of *B. subtilis* subsp. *subtilis* RUTs001, *B. subtilis* RUTs002, and *B. subtilis* RUTs003 in inhibiting the mycelial growth of *G. boninense* was assessed using the dual culture technique. Initially, *G. boninense* was cultured on PDA for a period of 10 days to establish a vigorous mycelial colony. Mycelial plugs, each 0.5 cm in diameter, were excised from the agar using a cork borer and transferred to new PDA plates, positioned 2 cm from the edge of the plate. These plugs were incubated at 28–32°C for 48 hours to initiate growth. Subsequently, a loopful of each *B. subtilis* strain, which had been cultured on nutrient agar (NA) for 48 hours, was streaked onto the opposite side of the plate,

2 cm from the edge, creating a confrontation zone with the fungal colony. For the control treatment *G. boninense* plugs were inoculated onto PDA plates without the addition of *Bacillus* spp. The experimental design adhered to a completely randomized design (CRD). The radius of the fungal colony was measured periodically and the percentage of inhibition was calculated using the following formula percent inhibition of radial growth (PIRG).

$$\% \text{PIRG} = (R1 - R2) / R1 \times 100$$

where R1 represents the average radius of the fungal colony in the control treatment, and R2 denotes the average radius of the fungal colony in the presence of the *Bacillus* treatment (Aydogdu *et al.*, 2021).

Efficiency of Bacillus spp. bioproducts in inhibiting mycelial growth of G. boninense in mushroom bag

The efficiency of the *Bacillus* spp. bioproducts was further evaluated in a mushroom bag system designed to simulate conditions relevant to oil palm cultivation. Both individual and combined formulations of the bioproducts were produced in the laboratory at a concentration of 1×10^9 CFU/g WP. A 100 g sample of each bioproduct formulation was mixed with 18 L of water to create a suspension. Additionally, a commercial biofungicide based on *B. subtilis* (Bio-Censer®, Thailand) was included as a reference treatment, applied at a standard dose of 50 g WP per 20 L of water. The cell suspensions were incubated overnight to enhance bacterial viability before being used to ferment a sawdust-based substrate for 10 days. This fermented substrate intended for *G. boninense* cultivation consisted of 100 kg of rubber sawdust, 6 kg of rice bran, 2 kg of calcium carbonate, and 200 g of magnesium sulfate. Each experimental comprised 700 g of composted mushroom per bag, which was pasteurized by steaming at 99 °C for 5–6 hours to eliminate contaminants. Once the substrate had cooled, *G. boninense* sorghum spawn was introduced into the mushroom bags. The bags were then incubated in a mushroom house maintained at a temperature of 30 °C, and the mycelial growth of *G. boninense* was measured at 7-day intervals until the substrate was fully colonized. The experiment was structured as a completely randomized design (CRD) with nine treatments and four replicates.

Results

The basal stem rot disease of oil palm

The isolation of the causal agent of basal stem rot disease from *Ganoderma* spp. fruiting bodies, followed by species identification using molecular techniques, confirmed the pathogen as *G. boninense* (Accession PP897285.1, 99.83% similarity). Morphologically, the mycelium of *G. boninense* on PDA exhibited a dark white coloration with a relatively coarse texture, characterized by densely interwoven hyphae. As the culture matured, the hyphae became increasingly sticky and underwent a gradual transition to a light brown hue. Full colonization of both Petri dishes and sorghum spawn was achieved within 15 days, reflecting the rapid growth potential of this pathogen. (Figure 2A-D).

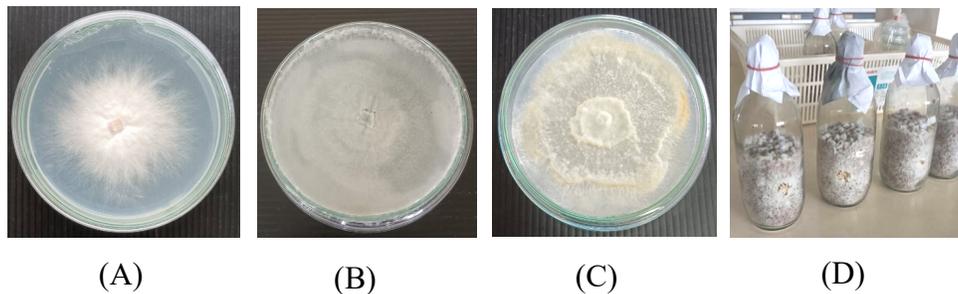


Figure 2. Mycelial of *G. boninense* on PDA at 5, 15 and 30 days (A, B and C), and spawn on the sorghum (D)

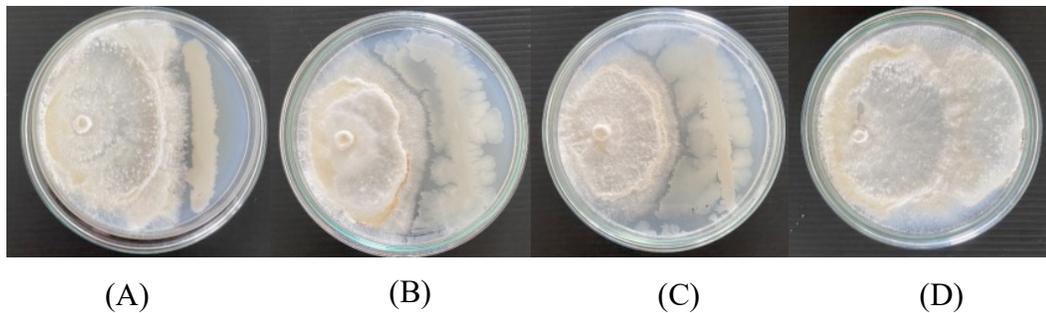
Efficacy of Bacillus spp. against G. boninense

The results of the dual culture technique demonstrated that the antagonistic *Bacillus* spp. effectively inhibited the mycelial growth of *G. boninense*, with varying degrees of efficacy among the strains. Specifically, *B. subtilis* RUTs003 exhibited the highest level of mycelial inhibition at 53.90%, followed by *B. subtilis* RUTs002 at 48.23%, and *B. subtilis* subsp. *subtilis* RUTs001 at 33.29% (Table 1 and Figure 3).

Table 1. Efficacy of bioproduct of *Bacillus* spp. for inhibiting mycelium growth of *G. boninense* by dual culture technique for 30 days

Treatments	Inhibition growth (%)				
	3 days	7 days	10 days	20 days	30 days
<i>B. subtilis</i> subsp. <i>subtilis</i> RUTs001	35.39±6.12 ^{b1/}	33.45±11.82 ^b	33.38±11.84 ^b	33.29±11.95 ^b	33.29±11.95 ^b
<i>B. subtilis</i> RUTs002	41.02±12.0 ^{ab}	48.36±8.63 ^{ab}	48.33±8.57 ^{ab}	48.23±8.60 ^{ab}	48.23±8.60 ^{ab}
<i>B. subtilis</i> RUTs003	47.36±2.01 ^a	54.11±2.39 ^a	53.92±2.66 ^a	53.90±2.42 ^a	53.90±2.42 ^a
Control	0.00±0.00 ^c	0.00±0.00 ^c	0.00±0.0 ^c	0.00±0.00 ^c	0.00±0.00 ^c

^{1/} = Same letters in the same column indicate that values are not significantly different ($p > 0.05$), mean compared by DMRT.

**Figure 3.** Mycelial of *G. boninense* inhibited by *B. subtilis* subsp. *subtilis* RUTs001 (A), *B. subtilis* RUTs002 (B), *B. subtilis* RUTs003 (C) and control (D)

Efficacy of Bacillus spp. bioproducts in inhibiting mycelial growth of G. boninense in mushroom bags

The duration required for *G. boninense* mycelial growth to achieve full colonization in the plastic bags varied significantly among treatments ($P < 0.01$). At 7 days after inoculation, the substrate fermented with the combination of *B. subtilis* subsp. *subtilis* RUTs001 and *B. subtilis* RUTs003 exhibited the slowest mycelial growth, measuring 1.65 cm, whereas other formulations resulted in growth ranging from 2.31 to 3.48 cm. This trend persisted at 14 and 21 DAI, with the combinations of *B. subtilis* subsp. *subtilis* RUTs001 + *B. subtilis* RUTs003, *B. subtilis* subsp. *subtilis* RUTs001 + *B. subtilis* RUTs002, and the commercial *B. subtilis* product recording mycelial growth of 6.03–8.37 cm, 6.18–8.55 cm, and 6.54–8.66 cm, respectively. By 28 DAI, the same pattern was observed, with

mycelial growth measurements of 11.79 cm, 11.78 cm, and 12.02 cm for these treatments, respectively. Full mycelial colonization of the substrate was achieved at 35 DAI for the combinations of *B. subtilis* subsp. *subtilis* RUTs001 + *B. subtilis* RUTs003 and *B. subtilis* subsp. *subtilis* RUTs001 + *B. subtilis* RUTs002, representing the slowest colonization period. This was followed by the commercial *B. subtilis* product, which reached full colonization at 34 DAI, while the untreated control and other formulations achieved full colonization at 32 DAI (Table 2). The characteristic mycelia of *G. lucidum* on the substrates at each stage are shown in Figure 4. The *G. boninense* did not produce fruiting bodies in this experimental setup, and the substrate began to degrade approximately after 4 months and completed degradation at 8 months. The substrates treated with all *Bacillus* fermented showed complete mycelial degradation, whereas control retained approximately 50% of *G. boninense* mycelium growth (Figure 5).

Table 2. Efficacy of biological product *Bacillus* spp. for controlling the mycelial growth of *G. boninense* in mushroom bag

Treatments	Mycelium growth (cm)				Mycelium full (days)
	7 days	14 days	21 days	28 days	
<i>B. subtilis</i> subsp. <i>subtilis</i> RUTs001	3.45±0.44 ^{a1/}	7.03±0.44 ^{cde}	9.01±0.12 ^{cd}	12.33±0.09 ^{ab}	32±0.00 ^c
<i>B. subtilis</i> RUTs002	3.48±0.27 ^a	6.98±0.30 ^{cdef}	8.81±0.21 ^{cde}	12.22±0.10 ^{ab}	32±0.00 ^c
<i>B. subtilis</i> RUTs003	2.89±0.30 ^b	7.29±0.60 ^{cd}	9.16±0.10 ^c	12.46±0.10 ^{ab}	32±0.00 ^c
<i>B. subtilis</i> subsp. <i>subtilis</i> RUTs001 + <i>B. subtilis</i> RUTs002	2.60±0.20 ^{bc}	6.18±0.38 ^{ef}	8.55±0.14 ^{ef}	11.88±0.12 ^b	35±0.00 ^a
<i>B. subtilis</i> subsp. RUTs001 + <i>B. subtilis</i> RUTs003	1.65±0.19 ^d	6.03±0.30 ^f	8.37±0.12 ^f	11.79±0.07 ^b	35±0.00 ^a
<i>B. subtilis</i> RUTs002 + <i>B. subtilis</i> RUTs003	3.45±0.07 ^a	8.78±0.16 ^a	11.88±0.19 ^a	12.97±0.07 ^a	32±0.00 ^c
<i>B. subtilis</i> subsp. <i>subtilis</i> RUTs001 + <i>B. subtilis</i> RUTs002 + <i>B. subtilis</i> RUTs003	2.94±0.21 ^b	8.13±0.52 ^{ab}	11.66±0.13 ^a	12.35±1.12 ^{ab}	32±0.00 ^c
<i>B. subtilis</i>	2.31±0.26 ^c	6.54±0.44 ^{def}	8.66±0.18 ^{def}	12.02±0.10 ^b	34±0.00 ^b
Control	2.68±0.19 ^{bc}	7.56±0.36 ^{bc}	9.63±0.31 ^b	12.59±0.19 ^{ab}	32±0.00 ^c

^{1/} = Same letters in the same column indicate that values are not significantly different (p > 0.05), mean compared by DMRT.

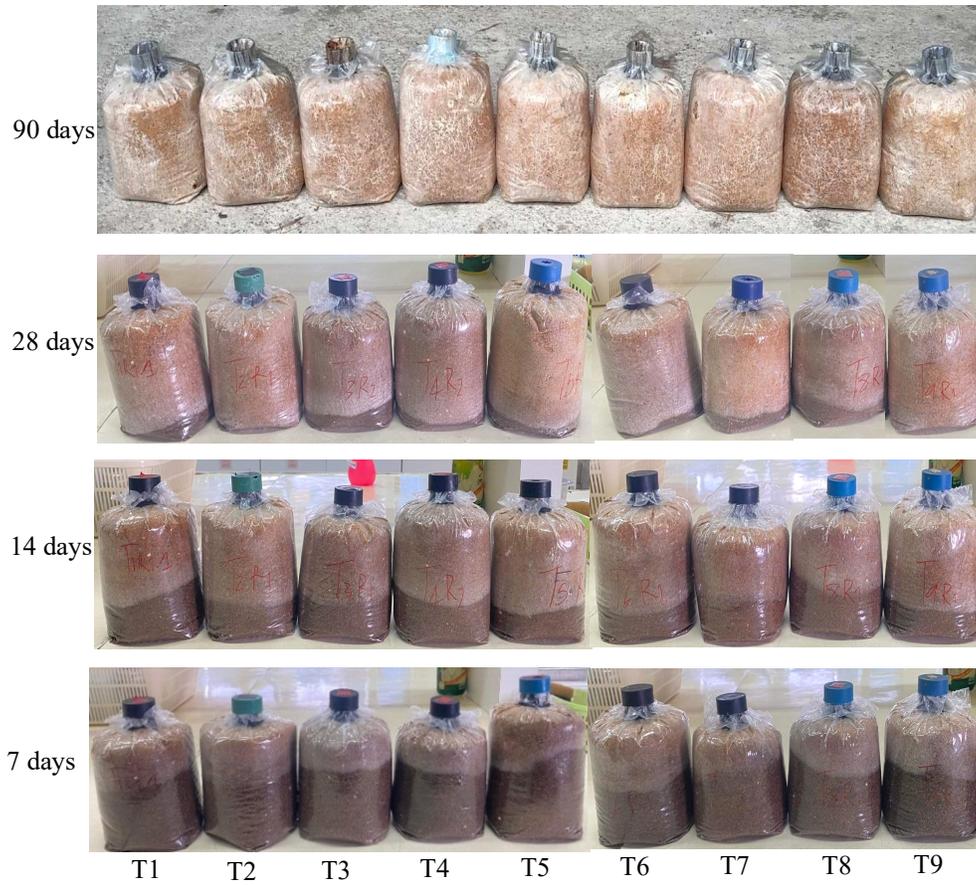


Figure 4. The mycelium growth of *G. boninense* in mushroom bag at 7, 14, 28 and 90 days after inoculation

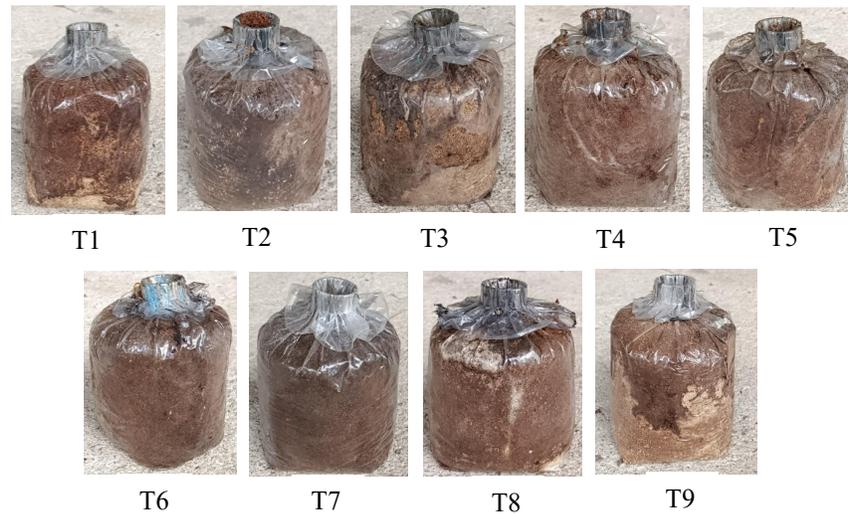


Figure 5. Colonization of *G. boninense* mycelium on a mushroom bag at 150 days after inoculation

Discussion

Presently, BSR disease poses a major threat to oil palm plantations particularly in southern Thailand, Indonesia, and Malaysia, where it directly contributes to significant reductions in yield. Haw *et al.* (2023) reported that BSR can result in yield losses of approximately 43%, with reductions exceeding 80% in severely affected plantations in Indonesia (Puspita *et al.*, 2019). In this study, the isolated *G. boninense* is believed to be the principal species responsible for the decline of oil palm trees, consistent with findings by Bharudin *et al.* (2022); Maherani *et al.* (2024); Widiantini *et al.* (2024). The progression of BSR disease was observed to occur in three distinct phases: the seedling stage (1–4 years), the mid-growth stage (6–12 years), and the mature stage (over 12 years) (Zakaria, 2023). In this investigation, the disease was occurred in an oil palm plantation approximately 8 years old, during its fruit-producing phase, leading to yield losses of up to 60%. Early-stage infections of *G. boninense* were symptomless, but subsequent symptoms included collapsing fronds, unopened spear leaves, yellowing, dieback, and basal rotting, with fruiting bodies emerging around the stem. Following infection oil palm trees declined rapidly, typically succumbing within 10 months, with severity peaking during the rainy season, which provides optimal conditions for fungal growth and dissemination.

The mycelial growth of *G. boninense* in the presence of these *Bacillus* isolates was significantly reduced compared to the control, with inhibition rates ranging from 33.29% to 54.11% over a 30-day period. These findings align with previous *in vitro* studies, which have reported the successful inhibition of *G. boninense* by bacterial biological control agents, including *Bacillus* spp. (Alexander and Chong, 2014; Alexander *et al.*, 2017; Nusaibah *et al.*, 2017), as well as other species such as *Streptomyces sanglieri* (Nur Azura *et al.*, 2016), *Burkholderia* sp. (Widiantini *et al.*, 2024), and *Pseudomonas aeruginosa* (Lim *et al.*, 2019).

The results showed that *Bacillus* had significant effect in suppressing mycelium growth of *G. boninense*. According to Alexander and Chong (2014); Alexander *et al.* (2017); Sapak *et al.* (2008); Supramani *et al.* (2022) reported that *Bacillus* inhibits the penetration of *G. boninense* in both nursery and the field condition. *Bacillus* is an endophytic bacterium, has the ability to penetrate vascular system. So that, its ability to competitive within the vascular system may restrict Ganoderma's access to both nutrients and space, thereby limiting its spread (Puspita *et al.*, 2019). Earlier laboratory and greenhouse investigations have listed the capacity of *Bacillus* to inhibit *G. boninense* mycelial development (Supramani *et al.*, 2022). Especially, *B. subtilis* has been shown to reduce BSR severity while concurrently promoting oil palm growth parameters and has potential as a biological resistance inducer for oil palm seedlings against *G. boninense* infection (Puspita *et al.*, 2019; 2020).

The mycelium growth of *G. boninense* on the substrates in this study indicated that endophytic *Bacillus* associated with *G. boninense* mycelium complete clearly mycelium degradation. While the control, which had considerable mycelial biomass remained about 50%. This pattern strongly suggests that the applied *Bacillus* direct mycolytic and antibiosis effects on *G. boninense* during substrate colonization, thereby reducing the practical inoculum of the basal stem rot disease (Ahmad *et al.*, 2023). The suppression is biologically reasonable because *Bacillus* spp. is widely recognized for producing extracellular cell-wall degrading enzyme and antifungal metabolites that can hyphal integrity and accelerate mycelial breakdown, ultimately limiting the persistence of wood decay pathogens in organic matter (Ahmad *et al.*, 2023; Rupaedah *et al.*, 2024). In addition, *Bacillus* products can provide antifungal activity *via* biosurfactant and lipopeptide-associated antagonism, which is compatible with strong inhibition and structural damage of fungal hyphae (Rupaedah *et al.*, 2024). According to Sitepu *et al.* (2022) reported that *Bacillus* can contribute both direct antagonism and host-mediated protection, depending on the application.

The results demonstrated that combined application of *B. subtilis* bioproducts effectively suppresses the mycelial growth of *G. boninense*.

Although the inhibitory effects were less distinct during the early stages. However, the combined application of *Bacillus* spp. bioproducts ultimately led to substantial suppression of *G. boninense* mycelial growth. The combinations of biocontrol agents differing in their mode of action especially bacteria which reside internally in the plant tissue without causing visible harm to their hosts can be used the disease management. According to Supramani *et al.* (2022) reported that the formulation of *B. subtilis* affect the incubation period and reduce the intensity of the disease. Alexander and Chong (2014) reported that elevated inoculum concentrations or synergistic combinations of *Bacillus* strains may enhance suppression of *G. boninense*.

The type of bacterium, active ingredients, nutrients, carrier material, and material ratios affect the performance of bioproduct bacteria. Puspita *et al.* (2019) reported that *B. subtilis* + solid + talc + tapioca flour was found as the best formula due to able control *G. boninense* by slowing down disease incubation period and reduce its intensity to 0% within 140 days. Accordance to this study, the formulation of *B. subtilis* including endospore of *B. subtilis* with addition of talcum + sCMS (Sodium carboxymethyl cellulose) with combined two isolates of *B. subtilis* in concentration of 1×10^9 CFU/ g WP resulting in the slowdown mycelium colonization and completely degraded within 150 days. This formula has been confirmed to reduce the disease incidence and severity index of green mold disease in *Ganoderma* mushrooms (Nuchaikaew *et al.*, 2024). While, Rajendran *et al.* (2018) reported that combination of biocontrol agents mixed with a talc-based formulation were developed and use to manage highly virulent, laccase producing soil borne pathogen. Which were informed that talc formulation combination with *B. subtilis* EPC5 showed high inhibition *G. lucidum* caused of basal stem rot disease of coconut. *G. boninense* is a soil borne and virulence pathogens hard for control. So that, next experiment should be test in the higher dose and study the use of combined bioproduct *Bacillus* by mixing with the planting material in seedling oil palm.

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Conflicts of interest

The authors declare no conflict of interest.

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