



# The Potential of *Bacillus* Spp. against *Pythium Myriotylum*, a Causative Agent of Rhizome Rot Disease in Ginger

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## Abstract

Ginger (*Zingiber officinale* Roscoe), a high-value cash crop in Malaysia is planted in large areas of Pahang and Sabah. The supply of good-quality ginger is limited due to rhizome rot caused by *Pythium myriotylum*. Chemical pesticides that is commonly used to control the disease has led to severe environmental impacts and causes adverse effects on consumer health. The present study aimed to use a safe and sustainable strategy to control the disease. *B. subtilis* that was determined as a phosphate solubilizing bacteria (PSB) when tested for their capability to solubilize phosphate on Pikovskaya media was also had an antimicrobial activity based on the antagonistic study against *P. myriotylum*. Evaluation under field environment showed that plants treated with *B. subtilis* was competitive with the commercial product (Bacillus Care) to control *P. myriotylum*. This was based on the significant lowest disease severity (36.25 %) in both *B. subtilis* and Bacillus Care-treated plants compared to the plants that treated with chemical fungicide; Mancozeb (40.0%) and untreated plants (50.0 %). The potential use of *B. subtilis* culture as a biostimulant and biocontrol agent against *P. myriotylum* could be enhanced by formulation in the near future to reduce the losses of harvest in ginger cultivation.

**Keywords:** Ginger; Rhizome rot; *Pythium myriotylum*; *Bacillus subtilis*; Bacillus Care

## Introduction

Ginger (*Zingiber officinale* Roscoe) that belongs to the Zingiberaceae family originates from Southeast Asia, including Malaysia. Ginger has been distributed to other countries due to its potential as an oriental spice that possesses various uses. In Malaysia, ginger is cultivated mostly on a small scale, whether as a single crop or interspersed with perennial crops like pineapple, rubber, and palm trees. Variety Bentong, which is normally grown in the highlands of Malaysia, is among the highest demand for local and international markets [1]. In terms of nutritional composition, the rhizome is rich with carbohydrates, vitamins, iron, and minerals like magnesium, chromium, and zinc. Unfortunately, the supply of good quality ginger is disrupted by several factors, including diseases due to the invasion of certain pathogens. Rhizome rot

disease is one of the most dangerous and destructive diseases of ginger [2]. This disease is prevalent in the ginger crop throughout the growing period, which contributed to a 50–90% reduction of its produce [2]. In Malaysia, the disease most likely occurred due to *Pythium myriotylum*. It takes a few months to infect the plant completely, which is totally different from bacterial wilt that has rapid infection. The wilt symptom started at the aerial leaves and end up in dry conditions. However, symptoms on the rhizome part are different, as no white ooze emits from the rhizome even though the rhizomes become soft and watery with creamy discoloration on the vascular system part. Rhizome rot caused by *P. myriotylum* is carried over and maintained through diseased rhizomes as oospores, which could be the source of primary inoculum. It has the availability to survive as saprophytes and decomposers as well as plant pathogens [3]. Chemical pesticides are commonly used to

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control rhizome rot disease throughout the planting cycle. Local farmers practice seed disinfestation by spraying with propamocarb as a fungicide. Mixture of Bordeaux and 0.25% benomyl are commonly used to produce the healthy seed meanwhile bromide dichloropicrin is used to treat the soil as a soil fumigant. Nevertheless, this practice has severe environmental impacts and causes adverse effects on consumer health that result from the accumulation of residue on the rhizomes and soils. With that concern, various strategies for human and environmental safety have been reported for controlling rhizome rot disease. Identification of resistant varieties towards *Pythium* spp. was done through conventional breeding. However, based on resistance screening by [4], only 7% of the 650 ginger accessions have the relative resistance to *Pythium* spp. Management of rhizome rot disease was reported to be effective using biological control agents in other countries [5]. Biological control is one of the sustainable and environmentally friendly approaches in agro ecosystems that may reduce the disease-producing ability or density of microbial inoculum. This is contributed by one or more organisms in their natural active state or through manipulation of the environment by mass introduction of antagonists [6,7]. *Bacillus* spp. that commonly refers as phosphate solubilizer is among those commonly used in the development of biocontrol products. Phosphate solubilization refers to the process by which insoluble forms of phosphorus are converted into soluble forms that plants can readily absorb. In soils, phosphorus is often present as calcium phosphate, iron phosphate, or aluminum phosphate, which are not directly usable by plants. Phosphate solubilizers are microorganisms that enhance phosphorus availability by converting these insoluble forms into soluble orthophosphate. Although phosphorus uptake can then lead to better plant growth and yield increment, its contribution in improving resistance to pests and diseases depends on the rhizosphere microbe growth and resistance. *Bacillus* spp. is involved in the production of antimicrobial compounds, such as H<sub>2</sub>S, HCN, and siderophore subtilin, surfactin, biofilm, difficidin, iturin, bacilysin, bacilomycin, and fengycin, that could inhibit the broad spectrum of plant pathogenic bacteria, fungi, and nematods [8]. In this study, *Bacillus* spp. was tested for their capability to solubilize phosphate and to control rhizome rot disease in laboratory and under field environment in MARDI Serdang Selangor.

## Methods

### Isolation of Rhizome Rot Causative Agent

A ginger plant that showed rhizome rot symptoms was collected at Millercler Nursery Seremban, Negeri Sembilan. The infected part of the rhizome was excised out and washed with 10% sodium hypochloride for two minutes, followed by 70% alcohol for another two minutes. The sample was then washed with sterile distilled

water twice. In a sterile condition, the sample was ground in 1 mL of distilled water with a mortar and pestle. One hundred µL of the ground sample was streaked on V8 (tomato juice) agar and incubated at 28 °C for 7 days. The morphology and microscopy examination of the respective pathogenic organism was observed under light microscope. Koch's postulate test was subsequently performed by drenching 10<sup>6</sup> spores/mL of pathogenic conidial suspension onto the soil of a healthy ginger plant. Plant was observed for ginger rhizome rot disease symptoms up to 4 weeks after inoculation.

### Molecular Identification of Pathogenic Fungus

PCR reaction was conducted to amplify the pathogenic fungus in accordance with the protocol provided by the manufacturer (Vivantis). Thirty µl of the total reaction volume consisted of 100 ng DNA genome template, 1.0 unit of Taq DNA polymerase (Vivantis), 1 X ViBuffer A, 3.0 mM MgCl<sub>2</sub>, 0.2 mM dNTP mix and 0.4 µM of universal primer; ITS1: TCC GTA GGT GAA CCT GCG G and ITS4: TCC TCC GCT TAT TGA TAT GC was conducted under the following conditions: initial denaturation (94°C for 3 min), followed by 30 cycles of denaturation (94°C for 30 sec), annealing (50°C for 1 min), extension (72°C for 30 sec) and final extension (72°C for 10 min). The PCR products with appropriate size was then sent for sequencing analysis. The derived DNA sequences were analysed using BLAST N for its homology identities compared to the sequences in National Centre for Biotechnology Information (NCBI).

### Evaluation of the Phosphate-Solubilizing Capability

*Bacillus subtilis* subspecies *spizizenii* used in this study was purchased from American Type Culture Collection (ATCC), while *Bacillus valesensis* used in this study was isolated from the rhizosphere of *Kaempferia parviflora* by another researcher. Ten (10) ml of both *B. subtilis* and *B. valesensis* were plated on Pikovskaya media. The petri plates were incubated for 14 days at room temperature (30 ± 1°C). The phosphate-solubilizing capability of pure bacteria was determined by a clear halo formed in the vicinity of the colony in triplicate.

### In vitro antagonistic test of *Bacillus* spp. against *P. myriotylum*

In vitro antagonistic tests were performed by the poisoned food technique in a CRD design twice with three replications. The antifungal agent or the extract is incorporated into the molten agar at a desired final concentration. In order to compare the performance of *Bacillus* isolates, a commercial product (*Bacillus Care*) recommended by the manufacturer to control *Pythium* spp. and a chemical-based fungicide (Mancozeb) were also tested in this study. Fifty (50) ml of *B. subtilis* and *B. valesensis* that had been

cultured overnight and 50 ml of Bacillus Care and Mancozeb (prepared according to the manufacturer's recommendation) were incorporated and mixed well in a conical flask containing 150 ml of sterilized V8 media. The mixture was then poured into a 9 cm-diameter petri dish and allowed to solidify thoroughly. Fresh-growing mycelium from the *P. myriotylum* plate was then cut (5 mm) with a scalpel and inoculated in the center of the Petri dish under aseptic conditions in laminar airflow. The mycelia growth of *P. myriotylum* was observed and measured for seven days. Inhibition percentage of radial growth (PIRG) for *P. myriotylum* mycelia in these treated media were calculated using the following formula:  $PIRG = ((rc - ri) / rc) \times 100$ , where *rc* denotes the mycelial growth in the control plate and *ri* denotes the mycelial growth in the treated plate. A V8 plate grown with fungus without any treatment was used as a negative control. Data was statistically analyzed using SAS 9.4 software to determine significant differences (Duncan's multiple range test,  $P < 0.05$ ). The most potent bacteria were selected for field plot testing.

### Evaluation on the effectiveness of *B. subtilis* against *P. myriotylum* in an open field plot

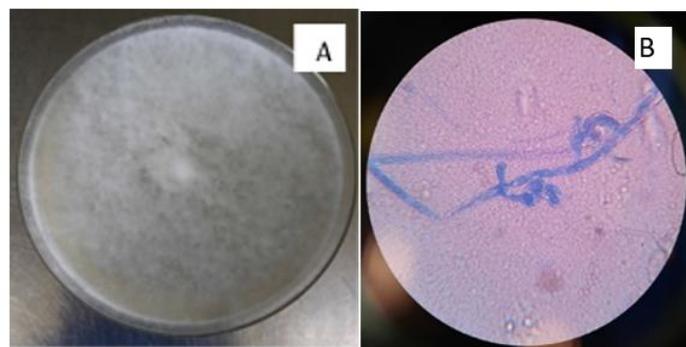
The field plot testing was conducted from November 2022 until April 2023 at MARDI Serdang (Latitude 11.152829°N; Longitude: 76.92°E) in a wet season. The rhizomes of ginger were planted in a polybag with cocoapeat media at a spacing of 30 x 20 cm. Each treatment consisted of five plants with three replications and laid in a randomized complete block design (RCBD). The plants were irrigated regularly using a fertigation system. The rhizome was soaked in 100 ml of cultured *B. subtilis*, *B. valsezensis* (0.5 McFarland standard was used), and a commercial product of Bacillus Care (prepared according to manufacturer recommendation) as a treatment prior to planting. The treatment was then repeated by drenching technique two weeks and two months after ginger was planted. Ginger plants that treated with chemical fungicide (Mancozeb) and water were used as a control plants. The severity of rhizome rot disease was visually rated based on the extent of yellowing symptoms of an affected area over the total foliage of the plants. The disease index percentage was measured as reported by [9] as follows: 0 = No infection, 1 = 1–25% infection / slight infection, 2 = 26–50% infection / moderate infection, 3 = 51–75% infection / slight severe infection, 4 = 76–100% infection / severe infection, 5 = dead. The calculation of disease severity percentage was calculated following the equation below and statistically analyzed using SAS 9.4:

$$\text{Disease severity percentage} = \left[ \frac{\sum (\text{Infected plant} \times \text{disease severity scale})}{\text{maximum scale} \times \text{Total number of plants}} \right] \times 100$$

## Results

### Identification of rhizome rot causative agent

The isolated causative agent of rhizome rot disease was observed having abundant fluffy white morphology with freely branching mycelium as hyaline and nonseptate on potato dextrose agar (Figure 1A). An observation under microscope found the fungus sporangia as filamentous and inflated (Figure 1B). Result of PCR confirmed that the causative agent of rhizome rot as *Pythium myriotylum*. The partial sequence of *Pythium myriotylum* isolate MDIPMyr has been register with accession number PQ815028 in NCBI. Result of Koch postulate test had confirmed the causative agent pathogenicity when  $10^6$  conidial spores/mL were inoculated onto a healthy ginger plant by drenching technique. Symptoms of rhizome rot disease by *P. myriotylum* were observed 14 days after inoculation on a healthy ginger plant (Figure 2). Initial symptoms of rhizome rot disease appeared on the aerial parts of the plant. It then progressed to younger leaves with similar symptoms. The appearance of brown lesions in the collar region of the pseudostem and chlorosis on the leaf showed the sign of rhizome rot. As the lesion enlarges, the stem started to rot and collapsed until the entire plant dies. Upon harvest, a soft, brown, water-soaked and decay symptom was observed on the rhizome.



**Figure 1:** Pathogenic fungi that cause rhizome rot disease. Colony morphology of pathogenic fungi on V8 media isolated from infected ginger plants (A). Microscopic observation of pathogenic fungal mycelium under 10X magnification (B).

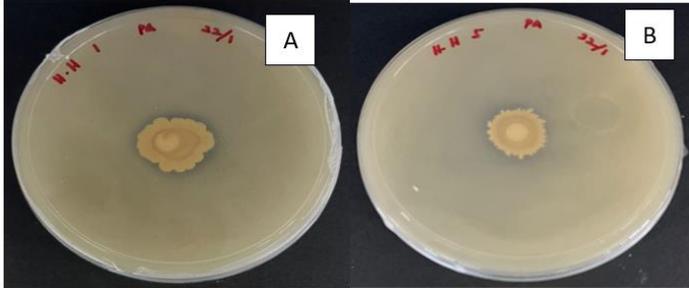


**Figure 2:** Koch's Postulate test. Symptom of wilting was observed approximately 2 weeks after the ginger plants was inoculated with *P. myriotylum*.

### Evaluation of the Phosphate-Solubilizing Capability

## SUNTEXT REVIEWS

The results for determining phosphate-solubilizing bacteria (PSB) was indicated by a clear zone around the colony 14 days after incubation at optimal temperatures (28–30 °C). Figure 3 shows the clear zone of both *B. valezensis* and *B. subtilis* on Pikovskaya agar medium. The clear zone indicates phosphate solubilizing activity. It reflects the efficiency of the bacteria in producing organic acids or enzymes that solubilize phosphate.



**Figure 3:** Phosphate solubilizing capability for *B. valezensis* (A) and *B. subtilis* (B) on Pikovskaya agar media.

### Antagonistic activity of *Bacillus* spp. against *P. myriotylum*

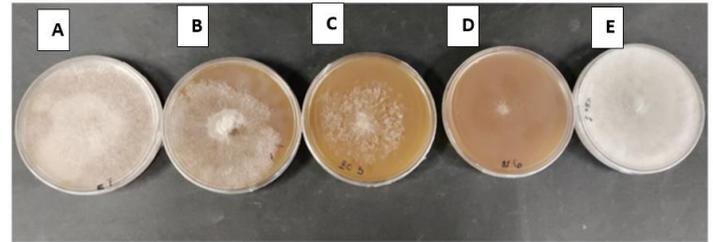
In addition, an antagonistic activity of *Bacillus* spp. was evaluated based on the mycelia growth of *P. myriotylum* on V8 agar by the poisoned food technique. Figure 4 shows the growth of *P. myriotylum* in media containing *B. valezensis* culture (A), chemical fungicide (B), commercial product Bacillus Care (C), *B. subtilis* culture (D) and control without any treatment (E).

The inhibition percentage of radial growth for *P. myriotylum* on V8 agar mixed with different treatments is tabulated in Table 1. *B. subtilis* was shown to have a significant inhibition percentage (80.04%) against the causal agent of ginger rhizome rot, *P. myriotylum*. This was followed by Mancozeb, which inhibited the growth of *P. myriotylum* by 45.03%, and the commercial product Bacillus Care (31.55%). Based on this result, *B. valezensis* was determined have no antimicrobial activity against *P. myriotylum*, although it showed the capability to solubilize phosphate.

### Evaluation on the effectiveness of biocontrol treatment against *P. myriotylum* in open field

In order to evaluate an effective treatment in the field, ginger was planted at a hot spot area where *P. myriotylum* was potentially infected the plant naturally without inoculation. *B. valezensis* that had no antimicrobial activity in the laboratory was omitted in the test. The severity of rhizome rot disease that was visually rated based on the extent of yellowing symptoms of an affected area over the total foliage of the plants showed that rhizome coated with *Bacillus* spp. in prior to planting and drenched with *Bacillus* spp. directly to the soil after planting was able to inhibit *P. myriotylum*. This was shown in Figure 5 by the lowest disease severity (36.25%)

in *Bacillus subtilis* and Bacillus Care-treated plants compared to the severity of plants treated with Mancozeb (40.0%) and control (50.0%).

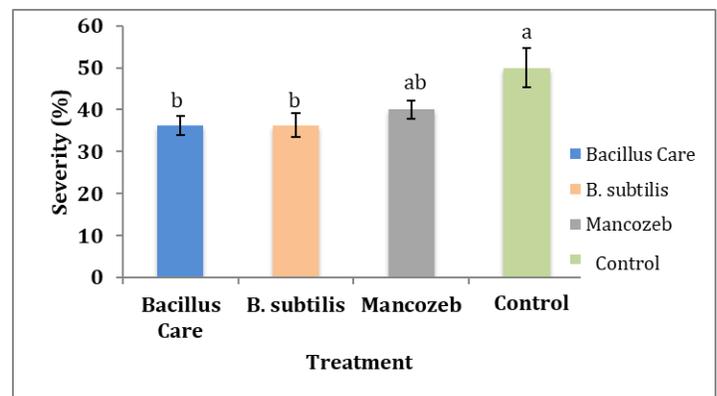


**Figure 4:** The growth of *P. myriotylum* in media containing *B. valezensis* culture (A), chemical fungicide (B), commercial product Bacillus Care (C), *B. subtilis* culture (D) and control without any treatment (E).

**Table 1:** Growth inhibition of *P. myriotylum* with different treatments in the laboratory. There is no significant difference ( $P < 0.05$ ) for mean inhibition with the same alphabet in the same column.

Treatment	PIRG (%)
Bacillus subtilis	80.04 ± 2.40 a
Bacillus valezensis	-1.54 ± 1.56 d
Bacillus Care	31.55 ± 2.53 c
Mancozeb	45.03 ± 3.25 b

\*Means covered by the different letters in a column is significantly different ( $P < 0.05$ ).



**Figure 5:** Mean of rhizome rot disease severity in ginger plant planted in the field with different treatments.

### Discussion

Rhizome rot disease is one of the major diseases in ginger worldwide. Identification of symptomatic ginger collected from Seremban, Negeri Sembilan Malaysia revealed *Pythium* spp. as the

major causative agent rather than *Fusarium* spp. It was mentioned by [10], at least fifteen *Pythium* spp. have been reported as ginger pathogens. *P. myriotylum* could be found distributed in warm regions worldwide. Many efforts have been taken place to overcome this disease including the use of *Bacillus* spp. as the biocontrol agents. Biological control agents are organisms that interact with the components of the disease triangle (host, pathogen, and environment). This refers to the purposeful utilization of introduced or resident living organisms by secreting substances that directly suppress the activities and populations of one or more plant pathogens. *Bacillus* species are known for their ability to solubilize phosphate as a vital nutrient for plant growth and development, integral to energy transfer, photosynthesis, and nutrient transport within plants. Phosphate solubilizer (PSB) is important to solubilize the reserves phosphate from soil and make it available to the plants, resulting in the promotion of plant growth and minimizing the application of phosphate fertilizer. According to [11], a significant positive effect of *B. subtilis* L2 as a bio-fertilizer was noticed to stimulate ginger growth when the suspension was used to inoculate onto the sterilized rhizomes of ginger. The capability to solubilize phosphate is vary depending on several factors, including the specific strain of *Bacillus*, composition of the soil or medium, and the incubation conditions. Differently to *B. valesensis*, the *B. subtilis* that determined as PSB by the clear zone on Pikoskaya media in this study was also had antifungal activity against *P. myriotylum*. The antifungal activity of the lipopeptides produced by *B. subtilis* subsp. *Spizizenii* of which the same strain used for this study was highlighted when tested against varies *Colletotrichum* spp. and *Fusarium* spp. fungal in different type of fruits [12]. Based on the field evaluation result, *B. subtilis* potential to control *P. myriotylum* was found to be comparable with the commercial product (*Bacillus Care*). This result was supported by a study of [13] who evaluated an inhibition of the *P. myriotylum* growth when the rhizome pieces had been pre-incubated with *Bacillus* sp. culture. The study also found an antifungal activity of *Bacillus* sp. WG4 when an effective protection to ginger rhizome was revealed upon inoculation with *P. myriotylum*. In other study by [14], an isolated *Bacillus subtilis* (BS-01) was found to produce a potent bioactive volatile organic compound that effective in inhibiting the growth of *Alternaria solani* in *Solanum lycopersicum*. We presumed the potential of *B. subtilis* used in this study could be more effective and enhanced by formulation rather than naked use without a protective capsule or outer membrane, which often makes them more vulnerable to environmental stresses. Formulation can protect the bacteria from environmental stresses, enhance survival rates, and improve their effectiveness in various conditions. Additionally, formulations often include carriers or additives that help with adhesion to surfaces, promote slow release, and provide nutrients that support bacterial growth. This was supported by [15], who mentioned that

formulation plays a significant role in determining the final efficacy of a *Bacillus*-based product, as do the processes of discovery, production, and stabilization of the biomass of the biocontrol agent. The potential of *B. subtilis* subsp. *Spizizenii* as a biostimulant and biocontrol agent in ginger need to be verified in a formulated form and tested for field application in the near future. Rather than that, combination with other methods such as cultural practice and chemical use also could be applied for best result.

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