

Granular biofertilizer amended with *Bacillus amyloliquefaciens* for growing yardlong bean (*Vigna unguiculata* subsp. *sesquipedalis*)

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Abstract

Yardlong bean (*Vigna unguiculata*) is a healthy vegetable that is popular in Southeast Asia. Farmers have frequently used pesticides while planting in order to avoid and eradicate pests. Utilising biofertilizer with microorganisms included to help with growth promotion and disease management is one such tactic. The aims of this study were to (1) produce granular biofertilizer amended with *Bacillus amyloliquefaciens* to control *Sclerotium rolfsii*-caused root and stem rot disease and (2) assess its effect on the growth of yardlong bean (*Vigna unguiculata* subsp. *sesquipedalis*). The bacterium which inhibited *S. rolfsii*, had the capacity to solubilize phosphate, enhanced the growth of yardlong bean and not antagonistic to *Rhizobium* TAL 638 was identified as *B. amyloliquefaciens* (HY 4-3-4). The granular biofertilizer that was created and included this bacterium (at 8.96 Log. number/g) continued to be efficient in inhibiting the mycelial growth of *S. rolfsii* even after 16 months of storage at room temperature (26–30°C). In the pot, granular biofertilizer was effective to control root and stem rot of the yardlong bean. The yardlong bean's stem length, fresh and dry weight, and number of nodules on the roots were all effectively increased by this granular biofertilizer. In the field, the granular biofertilizer was as effective as a chemical fungicide (mancozeb) to control root and stem rot of the yardlong bean. Thus, employing biofertilizers contributes to environmental sustainability by lowering the amount of chemicals used in growing yardlong bean.

Introduction

One vegetable in the Fabaceae family, the yardlong bean [*Vigna unguiculata* spp. *sesquipedalis* (L.)], is distinguished by its long, draped pods and ascending growth habit. Originally planted in West Africa, this bean is currently produced extensively across Southeast Asia, including Thailand, Malaysia, the Philippines, Indonesia, and Indonesia. It may be grown there year-round. (Benchasri & Bairaman, 2010; Benchasri et al., 2012).

The yardlong bean is nutritious containing vitamin A and C, protein, fiber, and other minerals including folic acid and vitamin B (Peyrano et al., 2016; Pandey et al., 2020). It's grown either as a stand-alone crop or as a crucial component in sequential and intercropping cropping systems. (Suma et al., 2021). One of Southeast Asia's top ten vegetables, particularly in Taiwan, southern China, and Bangladesh, is said to be the yardlong bean. In the Philippines, it is the most extensively cultivated legume and is referred to as "poor man's meat." Introduced to many lowland tropical regions, yardlong beans are often a minor garden produce. Grown as a summer crop in California and parts of Europe, it has gained popularity in the Caribbean and is particularly valued as a greenhouse vegetable. In Bangladesh, yardlong bean cultivation and consumption were encouraged to enhance human health and nutrition and to help avoid major health issues including diabetes, obesity, and several malignancies (Benchasri et al., 2012; Quamruzzaman et al., 2022).

In Thailand, the yardlong bean has been widely grown as a commercial crop for both internal use and export as fresh and frozen pods. The pods are frequently used in Thai cooking and are consumed both raw and cooked. They are a popular ingredient in the famed "Som Tum" salad as well as several other hot

cuisines as a side cooling vegetable. Both stir-fries and curries employ the beans. (Center for Agricultural Information, Office of Agricultural Economics, 2018). In 2018, approximately 8,925 ha of the yardlong bean have been grown by farmers in 73 provinces, with the total yield of the bean at approximately 63 tonnes. There are top five provinces, such as Phetchaburi, Ratchaburi, Pathum Thani, Surat Thani and Chon Buri, where the yardlong bean has been produced in the country (Information Technology and Communication Center, Department of Agricultural Extension, 2019).

Growing the yardlong bean requires frequent application of numerous pesticides because farmers perceive both insect pests and plant diseases as the major threat to their crop productivity (Bashir et al., 2002; Uddin et al., 2013). In Thailand, bean pod borer (*Maruca vitrata*) and aphids (*Aphis craccivora* and *Aphis glycines*) were the major insect pests, whilst leaf rust (*Uromyces vignae*) and damping off were important diseases of the yard long bean in Thailand (Schreinemachers et al., 2014).

The heavy reliance on application of synthetic pesticides to control pests of the yardlong bean has brought about the need to consider adopting other management practice and production standard ((Benchasri & Bairaman, 2010; Benchasri et al., 2012). Farmer bean aphid control prevention Synthetic chemicals are often preferred because they are readily available, convenient, and able to eliminate insects at all growth stages (Aktar et al., 2009). However, there are many consequences such as negative effects on user health, residues and accumulation in the produce and the environment. It kills useful animals and organisms and reduces the diversity in the ecosystem (Relyea, 2005; Sarwar, 2015; Sharma & Singhvi, 2017). According to the research report of Sarwar & Salman (2015), it was found that insect pests have a mechanism for creating chemical resistance through morphology, physiology, and behaviour adaptation. There are genes that can create resistance to chemicals. According to the research report of Pérez et al. (2000), it was found that vegetable pests can create resistance to insecticides, the toxicity value (LC_{50}) was higher than the comparison group.

The development of pests resistance to pesticides and deleterious effect to health have prompted the Ministry of Agriculture and Cooperatives (MOAC) of the Royal Thai Government to introduce the Thai “Q” Good Agricultural Practice (Thai Q GAP) for the production of fresh fruits and vegetables (National Bureau of Agricultural Commodity and Food Standards, 2018.). This move is to support the government policy to make Thailand as “kitchen of the world” (Ratanakreetakul & Korpraditskul, 2015). This standard has been used as a key tool for the farmers producing yardlong bean and other vegetables to access both domestic and international markets (Ratanakreetakul & Korpraditskul, 2015).

Integrated pest management (IPM) was reported to be effective to control pests in yardlong bean more than conventional pest control (Malacrinò et al., 2020). Despite yields being as high as in traditional production, IPM's expenses were greater than those of conventional management, which led to poorer profitability in the IPM (Malacrinò et al., 2020). The low profitability of IPM, in conjunction with the requirement to have knowledge associated with IPM application, may hinder its adoption by these farmers.

The technique to control plant pests that mitigate the risk of an increased pesticide residual on the produces should enhance the capacity of the farmers to comply with the Thai Q GAP. The research at the Faculty of Agricultural Technology, Songkhla Rajabhat University involves the production of organic fertilizers which are appropriate for use to produce fruits and vegetables. These organic fertilizers will not incur the increased nitrate level in the vegetables (Aires et al., 2013; Liu et al., 2014; Cintya et al., 2018). To enhance their use, they have been developed as a carrier of biological control agents. The organic fertilizers amended with the effective biological control agents should have the desired characteristics in both promoting plant growth and controlling soilborne diseases.

The objectives of this work are: (1) to isolate and screen for the bacterium that is antagonistic to *Sclerotium rolfsii* and promotes growth of the yard long bean and (2) to produce and evaluate the efficacy of the granular biofertilizer, amended with the antagonistic bacterium in both the greenhouse and the field condition.

Materials and Methods

Soil sample collection

Soil samples from four provinces, such as Phatthalung, Songkhla, Satun, and Nakhon Si Thammarat (with ten fields for each province), where yardlong bean (*V. unguiculata* subsp. *sesquipedalis*) had been grown, were used to isolate the bacteria. Since there had been no reports of yardlong bean root rot or stem rot disease caused by *S. rolfsii*, these fields were selected as sites for soil sampling.

Five sites within a 30x30 m area were selected to provide 500 g soil samples for each field. These samples were then combined in plastic containers. Following air drying, these soil samples were kept at room temperature (26–32°C) in plastic containers, and used for isolating *Bacillus* spp.

Isolation and selection of the bacteria

Two hundred ninety-five isolates of the bacteria were separated with a soil dilution plate technique as described by Kanjanamaneesathian et al. (1998). In a test tube, 1 g of soil sample was aseptically transferred to 9 mL of sterile distilled water. After that, these tubes spent 20 min in an 80°C water bath. One mL aliquots of the soil suspension, which had been serially diluted to 10^{-5} , were dispensed on Glucose soybean medium (GSM) in Petri dishes. After that, an aseptic L-shaped glass rod was used to distribute this soil solution. Following that, the plates were incubated for seven days at room temperature (26–32°C). For additional testing, a single colony of the bacteria cultured on GSM agar was obtained and transferred to test tubes containing potato dextrose agar (PDA) slant.

One bacterium, which was effective in inhibiting the mycelial growth of *S. rolfsii*, solubilized phosphate, promoted growth of the yardlong bean in the nutrient solution, and not antagonistic to the growth of *Rhizobium* sp. strain TAL 638, was identified using molecular technique as described below (White et al., 1990; Albores et al., 2014; Nyongesa et al., 2015).

Identification of the bacterium

Based on the morphological traits and appearance of the colonies, the bacterium was identified. (Albores et al., 2014; Nyongesa et al., 2015).

Only *Bacillus* spp. strain HY 4-3-4 which inhibited *S. rolf sii*, had the capacity to solubilize phosphate, enhanced the growth of yardlong bean and not antagonistic to *Rhizobium* TAL 638 was identified using molecular technique. The bacterium was subculture onto PDA in Petri dishes. This culture was incubated for seven days at room temperature (26–32°C) on a lab bench. After that, the pure culture was refrigerated at 4°C in order to facilitate DNA sequencing for further identification.

Sequence analysis of the ribosomal DNA (rDNA) internal transcribed spacer (ITS1 and ITS2) sections was used for molecular identification. From the bacterium's cells, genomic DNA was extracted using the QIAamp® DNA Mini Kit (Qiagen) technique.

PCR was performed using a forward primer sequence to amplify the internal transcribed spacer regions 1 and 2 of the rRNA gene cluster. ITS-1 5'-TCCGTAGGTGAACCTGCGG-3' and the reverse primer sequence IT-4 5'-TCCTCCGCTTATTGATATGC – 3' (White et al., 1990).

A total of 50 µL was used for the PCR amplifications, which involved combining 200 ng of the template DNA with 0.6 µM of each primer and 25 µL of 2x GoTaq® Green Master Mix. The PCR thermal cycle consisted of a 2 min initial denaturation at 95°C, 30 cycles (30 sec at 94°C, 30 sec at 55°C, and 2 min at 72°C), and a 5 min final extension at 72°C.

By using electrophoresis in 1.5% (wt/vol) agarose gel in 1× TBE buffer, aliquots (3 µL) were analyzed. Following a comparison of the sequencing data with the GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST/>), homology between the PCR fragments and sequences stored in the GenBank database was found using a nucleotide blast tool.

The *Bacillus* spp. strain HY 4-3-4 was determined to be *B. amyloliquefaciens* with 100% similarity (accession number JF899277.1). This bacterium was used to compare with other candidates, which have not been identified to the specific level, throughout this experiment.

Source and preparation of the inoculum of *S. rolf sii*

The yardlong bean plants with stem rot diseased symptom were collected from the farms in Phatthalung, Songkhla, Satun, and Nakhon Si Thammarat provinces. The diseased tissues (3x5 mm) containing both green and necrotic lesion were excised from these diseased plants. After that, these tissues were sterilized for 2 min in 1% sodium hypochlorite solution, twice washed in sterilized distilled water (SDW), and dried using sterilized filter paper. Once on sterile PDA (HiMedia, Mumbai; 39.0 g PDA powder and 1,000 mL of distilled water), the sterile tissues were aseptically transferred and incubated

for three days at 26–32°C. After being incubated, the developing mycelia were aseptically placed in test tubes onto PDA slants.

Pathogenicity test

After being cultivated on PDA in Petri plates, the pure culture of *S. rolfsii* was separated and allowed to incubate for 7 days at room temperature before producing sclerotia. These fungal cultures were used to inoculate the yardlong beans. The yardlong beans [(cv. Keaw Dok 5) grown in the mixture of soil, sand, and coconut coir at the proportion of 1:1:1 (v/v/v)] were inoculated with the fungus 15 days after sowing (DAS).

The inoculation was carried out by placing an agar piece (5x5 mm) containing three sclerotia on the soil surface at the base of a yardlong bean stem. The inoculated yardlong beans were watered daily and disease symptoms were recorded (7 days when the disease symptom will appear after inoculation). The pathogen was re-isolated using the diseased tissues, and Koch's postulation was fulfilled by keeping the pure culture of the re-isolated fungus in test tubes on PDA slant.

Antagonism test between the bacteria and *S. rolfsii*

Bacteria that effectively inhibited *S. rolfsii*'s mycelial growth were selected using a dual culture approach. A *S. rolfsii* agar plug was positioned in the middle of the PDA plate, and a pure culture of the bacteria was injected midway between the petri dish's rim and the *S. rolfsii* plug, forming a triangle. After 48 h of incubation at room temperature (26–32°C), a clear zone between the bacteria and *S. rolfsii* was evaluated. The ability of the bacterial isolates to form a clear zone larger than 6 mm was evaluated in order to assess their phosphate solubilizing capacity.

Phosphate-solubilizing test

A pure culture of the bacteria, selected based on mycelial inhibition, was inoculated halfway between the rim of the petri-dish with triangular formation on double layered plate incorporated with calcium phosphate using a modified medium containing per liter of distilled water: glucose 10 g, yeast extracts 0.50 g, CaCl₂ 0.10 g, MgSO₄.7H₂O 0.25 g, 10% CaCl₂ 30 mL, 10% K₂HPO₄ 20 mL, and agar 17 g (in solid medium) pH 7.0 (Malboobi et al., 2009). At room temperature (26–32°C), the inoculated plates were incubated.

After four days, the diameter of the bacterial colony and the zone of clearing (halo) surrounding it were measured in triplicate. By dividing the colony's diameter (cm) by the halo's diameter (cm), the Phosphate Solubilization Index (PSI) was computed (Kumar & Narula, 1999). *Rhizobium* sp. strain TAL 638 was tested for antagonistic activity against *Bacillus* spp. isolates whose PSI was more than 2 cm.

Antagonism test between selected bacteria and *Rhizobium* sp. strain TAL 638

The bacteria, that were chosen based on their antagonistic activity against *R. solfsii* and phosphate solubilizing capacity, were employed in the cross-streak method to ascertain their antagonistic activity against *Rhizobium* sp. strain TAL 63.

The PDA plate was streaked with each isolate of the chosen bacteria, and then *Rhizobium* sp. strain TAL 638 was inoculated by cross-streaking. After incubation for 72 h at room temperature (26–32°C), the antagonistic activity of each bacterial isolate against *Rhizobium* sp. strain TAL 638 was evaluated. The following describes how the isolates of the selected bacteria, which did not inhibit growth of *Rhizobium* sp. strain TAL 638, were assessed for their ability to promote the growth of yardlong beans in Somasegaran solution in a laboratory setting (Somasegaran & Hoben, 1994).

Growth promotion test using nutrient solution

One hundred seeds of yardlong beans (cv. Keaw Dok 5) were surface sterilized in 20% clorox solution 100 mL for 3 min, followed by rinsed thoroughly in sterile water 100 mL for 3 min. These seeds were placed between two sheets of sterile water-soaked paper and put into a tray (dimension 24.5x34.5x5.5 cm). The seeds, which had been wrapped between these papers in a tray, were incubated in a dark cabinet at room temperature (26–32°C).

After seven days, two healthy seedlings of the yardlong bean were transplanted to a sterile plastic bag which contained 200 mL somasegaran solution (pH 6.8). To assess if microorganisms are successful at promoting plant growth, the cotyledons of the seedlings were removed after growing them in the solution for 7 days. These seedlings were inoculated with both *Rhizobium* sp. strain TAL 638 and the selected bacterium (using one mL for each organism at 9.00 Log. number/mL) by using a syringe to transfer them into the nutrient solution.

After growing the seedlings for 30 days, the length of the shoot, fresh weight, and dry weight of yardlong bean of each plant were assessed. There were five replications for each isolate of the selected bacterium. The plants that were not infected and those that received 0.05% KNO₃ were utilized as the negative and positive controls, respectively. Each treatment consisted of 10 replications.

Pot test in the greenhouse

Experimental design

In the pot test, five bacterial isolates that grew the yardlong bean in the nutrient solution both synergistically and antagonistically against *Rhizobium* sp. strain TAL 63 were employed. A completely randomized design (CRD) was used in the arrangement of the experiment. The control treatment consisted of plants that were only infected with *S. rolfsii*. Each treatment consisted of 10 replications, each including 4 plants in a pot.

Pot preparation and pathogen inoculation

Loam (soil texture: 40% sand, 40% silt, 20% clay, pH 6.5, EC 65.50 $\mu\text{S}/\text{cm}$), cow manure, and coconut coir at the proportion of 3:1:1 (V/V/V) were used to prepare a “material used to grow plants”. The mixture was thoroughly mixed and dried outdoor for 7 days. The plastic pots, which were 25 cm in diameter and 21 cm in height, were filled with this dry mixture, after which the agar plugs of *S. rolfsii* culture on PDA containing 3 sclerotia were placed at a depth of 1.0 cm of the mixture in the centre of the plastic pot for 24 hours before sowing the yardlong bean seeds.

Seed preparation and planting

One hundred seeds of yard long beans (cv. Keaw Dok 5) were surface sterilized in 20% clorox solution 100 mL for 3 min, followed by rinsed thoroughly in sterile water 100 mL for 3 min. These seeds were then soaked with the bacterial suspension 1 mL containing both *Rhizobium* sp. strain TAL 638 and selected bacterium (prepared from using 1 mL for each organism at 9.00 Log. number/mL) for 24 h. Four seeds of the yardlong bean were planted in the centre of each plastic pot adjacent to where the agar plug of *S. rolfsii* had been placed. Only one healthy plant was maintained by removing the other three seedlings 7 days after planting. Sprinklers were used twice a day, in the morning and the afternoon, to water the plants.

Data collection

Seven days after sowing, the percentage of yard long beans that survived was noted. 40 days after planting, the growth assessment in the greenhouse testing was completed. The distance between the top leaf and the soil surface was used to measure plant height. Yardlong beans were evaluated for both fresh and dry weight using the above-ground portions. Roots of yard long bean were washed to count number of nodules per plant. The material used to grow plants was sieved through a 2 mm sieve to obtain the sclerotia (Rodriguez-Kabana et al., 1974), after which the number of sclerotia per pot was counted.

Preparation of granular biofertilizers amended with the bacterium

Five different ingredients were used to prepare each granular biofertilizer (either PB 1-2-4, *B. amyloliquefaciens* (HY 4-3-4), or RN 1-2-4). The main components of granular biofertilizer composed of soil mixture with *Samanea saman* leaves 600 g, cow manure 150 g, coconut coir fiber 150 g, and clay 100 g. Each ingredient used to prepare the granular biofertilizers was sieved through a 2 mm pore size and sun dried for 7 days. Each sieved ingredient was weighted and mixed thoroughly with a mixer for 15 min after which molasses 10 mL, and spore suspensions of the selected bacterium 600 ml (9.56 Log. number/mL), prepared as described by Wiwattanapatapee et al. (2004), were added.

The mixed materials were then extruded through the extruder (Anek Karnchang Co., Ltd, Thailand) (5 mm pore size). The extruded moist granular biofertilizers were dried in the open air for 48 h. The finished granular biofertilizer, supplemented with the selected bacteria, was kept in a plastic bag at room temperature (26–32°C) for further investigation.

Enumeration of viable bacteria in the granular biofertilizers

Viable bacteria in the granular biofertilizer were enumerated using the drop plate technique. (Zuberer, 1994). The viability tests were performed after the granular biofertilizer was kept in plastic bags at room temperature (26–32°C) for 2, 4, 6, 8, 10, 12, 14, and 16 months.

A suspension of the granular biofertilizer in sterile distilled water at the proportion of 1:99 (w/v) was prepared. One gram of granule biofertilizers was suspended in 100 mL of distilled water at room temperature (26–30°C). The granules biofertilizer suspension was agitated at 200 rpm until the granules had entirely dissolved. To destroy the vegetative cells, the dissolved granules biofertilizer suspension was incubated in an 80°C water bath for 20 min.

The viable bacteria were then grown in PCA at room temperature (26–32°C) for 18–24 h, following which colony forming units were counted. The viable bacteria value (Log. number/g) was calculated using 6 replications (6 drops) each dilution.

Testing the efficacy of the granular biofertilizer on growth promotion and disease suppression in the greenhouse

The preparation of planting material, pathogen inoculum and inoculation, seed preparation and planting for the efficacy test in the greenhouse test were similar to that which were described for the pot test, except that the yard long bean seeds were soaked only with 1 mL *Rhizobium* sp. strain TAL 638 (9.00 Log. number /mL) for 24 hours.

Before sowing these rhizobial treated seeds, ten g of each granular biofertilizer formulation were applied in the hole (at the depth of 5 cm). Plants were irrigated with a sprinkler every morning and afternoon.

The experiment was conducted using a completely randomized design (CRD) with five treatments. Treatments included (1) nil control (yardlong beans that were not inoculated with *S. rolf sii*), (2) control (yardlong beans that were inoculated with *S. rolf sii*), (3) PB 1-2-4 granular biofertilizer, (4) *B. amylo liquefaciens* (HY 4-3-4) granular biofertilizer, and (5) RN 1-2-4 granular biofertilizer. Treatments (3)-(5), yardlong beans were inoculated with *S. rolf sii*. Each treatment consisted of 10 replications with four plants in each pot.

Percentage of survival of yardlong beans was recorded 7 days after planting. The greenhouse growth evaluation was performed 40 days following the planting. Plant height was measured as described above. Fresh and dry weight were determined using above-ground parts of yardlong beans.

The roots of each sampled yardlong bean were first put in wash basin and washed with high-pressure water in the laboratory. Tweezers were used to pick out the remaining soil particles from each root. Any nodules found on long bean roots were separated and counted. The number of sclerotia of *S. rolf sii* was counted after they were sieved through a 2 mm mesh and separated them from the material used to grow plants.

Testing the efficacy of the granular biofertilizer in the field

The study was conducted at the Faculty of Agricultural Technology, Songkhla Rajabhat University, Muang, Songkhla, Thailand (Latitude 7° 00' 14.20" N Longitude 100° 30' 1.75" E Altitude 56 m above sea level). The yardlong bean plots were ploughed, and beds (1.0 m width, 9.0 m length, and 0.20 m height) were prepared for planting. Each bed was planted with two rows of yard-long beans (80 cm between rows, 50 cm between plants). The soil texture was sandy loam, with 2.52% organic matter, 0.06% nitrogen, 153.23 mg/kg available phosphorus, 96.35 mg/kg potassium, and a pH of 5.6.

The trial followed a randomized complete block design (RCBD), with 4 treatments and 5 replications. The treatment consists of (1) nil control (yardlong beans that were not inoculated with *S. rolfsii*), (2) control (yardlong beans that were inoculated with *S. rolfsii*), (3) chemical fungicide (yardlong beans that were sprayed with mancozed fungicide at the rate of 50 g per 20 L of water one day after inoculated with *S. rolfsii*), and (4) granular biofertilizer (ten grams of granular biofertilizer were mixed in the soil before sowing; yardlong beans that were inoculated with *S. rolfsii*).

The preparation of the yardlong bean seeds and planting for the field test followed the procedure specified in the greenhouse. The yardlong beans in the field, however, received chemical fertilizers (15N-15P-15K) thrice. The first application was carried out at the time of sowing the seeds (at 5 g per plant), followed by the second time at 20 days after sowing (at 10 g per plant) and the third time at 55 days after sowing (at 15 g per plant). The yardlong beans were regularly watered with sprinkler (15 min) twice a day (Early in the morning and early in the afternoon, except on rainy days).

Data collection

Data on the percentage of survived seedling was collected 7 days after sowing. After sowing the yardlong beans for 70 days, the pod of the yardlong beans were harvested to collect the data on the pod length, fresh pod weight per plant and number of pods per plant.

Statistical Analysis

Data were analyzed using one-way ANOVA and compared with Duncan's Multiple Range Test (DMRT) at $P < 0.05$ and $P < 0.01$.

Results

Isolation and identification of the agent causing root rot in yardlong beans

Yardlong bean root rot was caused by *S. rolfsii*, which was identified based on its morphological characteristics. (Prasad, 2012; Paparu et al., 2020). The mycelium was colourless, transparent, thin-walled, and pure white with distant septate hyphae; after 4 days, the colony's colour changed to silky

white. The sclerotia were formed as small white spherical granules and enlarged to dark brown after 7 days of culturing (Fig. 1)

The *S. rolfsii* was proved to be pathogenic to 15-day-old yardlong beans. This pathogen caused yellow lesion to the yard long bean stalk 4 days after inoculation and this yellow lesion turned to dark brown. The infected stalk was rotten and decay 10 days after inoculation. This diseased tissue was used to isolate the pathogen and the pure culture of *S. rolfsii* was subsequently obtained to complete the Koch's postulation.

Isolation of antagonistic and phosphate-solubilizing bacteria

This investigation obtained 295 isolates of *Bacillus* spp. (Table 1). Among 295 *Bacillus* spp. isolates, 20 isolates inhibited the mycelial growth of *S. rolfsii* by establishing a clear zone of more than 6 mm (Fig. 2). The LN1-3-5 isolate had the highest inhibition efficiency (Table 2). Among these isolates, 12 isolates were phosphate solubilization on double layered plate incorporated with calcium phosphate. The RN 1-2-4 isolate had the highest solubility efficiency (Table 2).

Table 1
The number of *Bacillus* spp. isolates inhibited mycelia growth of *Sclerotium rolfsii*, isolated from planted Yard long bean soil sources

Soil sources	Plot code (No.)	Number of isolates
Pa Bon, Phatthalung	PB (1)	8
Tamot, Phatthalung	TM (3)	24
Kong Ra, Phatthalung	GR (1)	6
Bang kaeo, Phatthalung	BK (2)	16
Hat Yai, Songkhla	HY (6)	48
Khuan Niang, Songkhla	KN (3)	20
Bang Klam, Songkhla	BKL (2)	17
Singhanakhon, Songkhla	SN (3)	22
Sathing Phra, Songkhla	SP (1)	10
Ranot, Songkhla	RN (3)	25
Chana, Songkhla	JN (1)	8
Rattaphum, Songkhla	RP (6)	48
Langu, Satun	LN (2)	10
Thung Wa, Satun	TW (1)	9
Khuan Don, Satun	KD (2)	6
Khuan Kalong, Satun	KL (2)	10
Chian Yai, Nakhon Si Thammarat	CHY (1)	8
Total	40	295

Table 2

In vitro reaction of some selected antagonistic bacteria against *Sclerotium rolfsii* in dual culture, and phosphate solubilization Index (PSI)

<i>Bacillus</i> spp. isolates	Width of clear zone (mm)	PSI
PB 1-2-4	8.38 ^c	2.08 ^{ab}
GR 1-3-4	7.21 ^d	-
TM 3-1-5	7.29 ^d	1.68 ^{bc}
HY 4-3-4	8.54 ^c	2.14 ^{ab}
HY 5-1-4	9.61 ^{ab}	1.48 ^{cd}
HY 5-2-4	9.82 ^{ab}	1.21 ^{cd}
HY 5-2-5	6.92 ^{de}	1.04 ^d
HY 6-1-4	9.59 ^{ab}	1.68 ^{bc}
HY 6-2-5	6.21 ^e	-
KN 1-3-5	6.13 ^e	2.18 ^a
KN 2-1-5	7.54 ^d	2.00 ^{ab}
KN 3-3-5	9.63 ^{ab}	1.22 ^{cd}
BKL 1-1-5	9.88 ^{ab}	-
BKL 2-3-5	9.00 ^{bc}	1.09 ^d
SN 1-1-4	9.42 ^{ab}	-
SN 3-2-5	6.25 ^e	-
RN 1-2-4	7.04 ^{de}	2.41 ^a
RN 1-4-4	6.67 ^{de}	-
LN 1-3-5	10.00 ^a	-
RP 2-3-5	6.96 ^{de}	-
F-test	**	13.60

** Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at $p < 0.01$

<i>Bacillus</i> spp. isolates	Width of clear zone (mm)	PSI
C.V. (%)	5.39	**
** Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at $p < 0.01$		

Antagonistic tests between *Bacillus* spp. and *Rhizobium* sp. strain TAL 638

From 12 isolates tested, PB 1-2-4, *B. amyloliquefaciens* (HY 4-3-4), KN 2-1-5, KN 1-3-5, and RN 1-2-4 isolates had no antagonistic activity against *Rhizobium* TAL 638.

Growth promotion in nutrient solution and pot tests

In the nutrient solution, *Rhizobium* strains TAL 638 and *B. amyloliquefaciens* (HY 4-3-4) promoted growth of the yardlong beans in somasegaran solution based on the assessment of dry weight (Fig. 3 and Table 3).

Table 3

The growth of yard long bean in somasegaran solution with inoculation of *Rhizobium* strains TAL 638 and *Bacillus* spp.

Solution	Stem length (cm)	Fresh weight (g)	Dry weight (g)
Somasegaran (negative control)	17.94 ^d	2.21 ^f	0.64 ^d
Somasegaran + KNO ₃ (positive control)	21.50 ^{cd}	2.64 ^e	0.63 ^d
Somasegaran + TAL 638	23.13 ^{bc}	1.45 ^g	0.41 ^e
Somasegaran + TAL 638 + PB 1-2-4	18.94 ^d	1.19 ^h	0.26 ^f
Somasegaran + TAL 638 + <i>B. amyloliquefaciens</i> (HY 4-3-4)	26.25 ^{ab}	4.44 ^a	1.99 ^a
Somasegaran + TAL 638 + KN 1-3-5	27.63 ^a	2.72 ^d	1.86 ^b
Somasegaran + TAL 638 + KN 2-1-5	24.00 ^{abc}	3.92 ^b	1.88 ^b
Somasegaran + TAL 638 + RN 1-2-4	26.21 ^{ab}	3.36 ^c	1.48 ^c
F-test	**	**	**
C.V. (%)	7.56	0.95	2.00
** Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at $p < 0.01$			

In the pot, *Rhizobium* strain TAL 638 and *Bacillus* spp. strains RN 1-2-4, *B. amyloliquefaciens* (HY 4-3-4) (21), and KN 1-3-5 promoted growth of the yardlong beans in the greenhouse (Table 4).

Table 4
Efficacy of *Bacillus* spp. in antagonistic and growth enhancement for Yard long bean in pot test

Treatment	Stem length (cm)	Fresh weight (g)	Dry weight (g)	Survival (%)	No. of nodules per plant	No. of Sclerotia
Control	128.75 ^b	63.58 ^c	10.65 ^b	25.00 ^c	18.00 ^d	250.00 ^a
TAL 638 + PB 1-2-4	168.25 ^{ab}	118.35 ^b	19.00 ^{ab}	81.25 ^a	114.00 ^b	39.00 ^b
TAL 638 + <i>B. amyloliquefaciens</i> (HY 4-3-4)	241.75 ^{ab}	177.22 ^a	30.77 ^a	68.75 ^{ab}	100.00 ^b	21.00 ^c
TAL 638 + KN 1-3-5	162.75 ^{ab}	147.14 ^{ab}	28.64 ^a	37.50 ^{bc}	160.00 ^a	45.00 ^b
TAL 638 + KN 2-1-5	176.00 ^{ab}	147.84 ^{ab}	26.75 ^a	56.25 ^{a^{bc}}	62.00 ^c	37.00 ^b
TAL 638 + RN 1-2-4	251.75 ^a	161.15 ^{ab}	31.71 ^a	68.75 ^{ab}	118.00 ^b	18.00 ^c
F-test	**	**	**	**	**	**
C.V. (%)	8.56	6.06	8.52	9.63	14.82	8.75
** Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at p < 0.01						

Rhizobium strain TAL 638 and three strains of *Bacillus* spp. (strains PB 1-2-4, and RN 1-2-4), and *B. amyloliquefaciens* (HY 4-3-4) were effective to control disease of the yardlong beans (Table 4).

Rhizobium strain TAL 638 with either *Bacillus* spp. strains KN 1-3-5 (160), RN 1-2-4 (118), PB 1-2-4 (114), or *B. amyloliquefaciens* (HY 4-3-4) (100) promoted the nodulation of the root in yardlong beans (Table 4).

Rhizobium strain TAL 638 with either *Bacillus* spp. strain RN 1-2-4 (18), *B. amyloliquefaciens* (HY 4-3-4) (21), strains KN 2-1-5 (37), or strains PB 1-2-4 (39) decreased the number of sclerotia per plant as compared to the nil control (250) (Table 4).

Enumerating viable bacteria in granular biofertilizers

The population of *B. amyloliquefaciens* (HY 4-3-4) remained very high at 10.90 Log. number/g, followed by that of PB 1-2-4 (10.49 Log. number/g) and RN 1-2-4 (10.77 Log. number/g) (Table 5). The number of *Bacillus* spp. in each formulation remained steady and high during the 16-month storage period (Table 5). The physical characteristics of the granules, such as texture and colour, were stable during storage in plastic bag at room temperature (26–32 °C).

Table 5

Number of viable bacterial antagonist in granular biofertilizer after storage at room temperature for 16 months

<i>Bacillus</i> isolates	Number of viable bacterial antagonist (Log. number/g)				
	0*	4	8	12	16
<i>B. amyloliquefaciens</i> (HY 4-3-4)	10.90	9.97	9.89 ^a	9.77 ^a	8.97
RN 1-2-4	10.49	9.47	8.92 ^b	8.81 ^b	8.46
PB 1-2-4	10.77	9.71	9.42 ^a	8.90 ^b	8.60
F-test	ns	ns	**	**	ns
C.V. (%)	3.96	3.87	2.89	2.59	4.68
* Months after storage at room temperature (0, 4, 8, 12, and 16 months)					
ns = means in the same column are not statistical significantly different					
** Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at $p < 0.01$					

Testing the effect of granular biofertilizer formulation on greenhouse test

Granular biofertilizer, amended with *Bacillus* spp. either PB 1-2-4, RN 1-2-4, or *B. amyloliquefaciens* (HY 4-3-4) was effective to not only protect the yardlong beans from infection, but also suppress growth of *S. rolfsii* (Table 6). Granular biofertilizer, amended with *B. amyloliquefaciens* (HY 4-3-4) was most effective in promoting the nodulation of the yardlong beans in the pot (Table 6).

Table 6
Efficacy of granular biofertilizer in the pot test

Treatment	Survival (%)	Stem length (cm)	Fresh weight (g)	Dry weight (g)	No. of nodules per plant	No. of Sclerotia
Control I (normal plant)	75.00 ^a	235.00 ^b	128.11 ^b	23.52 ^b	105.00 ^c	0.00 ^b
Control II (inoculated with <i>S. rolfsii</i>)	18.75 ^b	65.00 ^c	23.82 ^c	4.28 ^c	10.75 ^d	321.50 ^a
PB 1-2-4	81.25 ^a	335.00 ^{ab}	189.13 ^a	29.59 ^{ab}	130.25 ^{ab}	32.75 ^b
<i>B. amyloliquefaciens</i> (HY 4-3-4)	87.50 ^a	355.75 ^a	190.35 ^a	36.07 ^a	142.50 ^a	12.75 ^b
RN 1-2-4	68.75 ^a	302.00 ^{ab}	185.77 ^a	32.33 ^{ab}	119.25 ^{bc}	28.75 ^b
F-test	**	*	*	*	**	**
C.V. (%)	6.23	6.86	13.04	13.53	9.11	15.57
* Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at $p < 0.05$						
** Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at $p < 0.01$						
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Testing the effect of granular biofertilizer formulation on field test

Granular biofertilizer, amended with *B. amyloliquefaciens* (HY 4-3-4), was effective to control diseases of yardlong beans, although it was less effective than mancozeb (Table 7). Both granular biofertilizer (amended with *Bacillus* spp. PB 1-2-4, or RN 1-2-4) and mancozeb had potential to increase yield of the yardlong beans in the field when pod length and pod weight were assessed (Table 7).

Table 7
Efficacy of granular biofertilizer in the field test

Treatments	Survival (%)	Pod length (cm)	Fresh pod weight per plant (g)	No. of pods per plant (pod)
Control I (normal plant)	57.50 ^c	57.53 ^b	148.67 ^a	8.06 ^b
Control II (inoculated with <i>S. rolfsii</i>)	0.00 ^d	0.00 ^c	0.00 ^b	0.00 ^c
Mancozed	85.00 ^a	60.53 ^a	152.84 ^a	10.79 ^a
Granular biofertilizer	71.25 ^b	60.85 ^a	170.56 ^a	11.14 ^a
F-test	**	*	*	*
C.V. (%)	10.05	3.71	12.91	8.38
* Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at $p < 0.05$				
** Means followed by the same letter are not significantly different by Duncan's Multiple Range Test at $p < 0.01$				

Discussion

Although several insect pests and plant diseases have been reported to cause economic loss to the yardlong bean in Thailand (Schreinemachers et al., 2014), *S. rolfsii* causes root rot and stem rot disease (Fig. 1) has been a severe problem in Songkla province (Sajeena et al., 2021; Kukulevithana et al., 2023). Our research has been initiated to address the local disease problem that might become the regional and national problem in the future because the pathogen has been reported to have a wide host range (Mullen, 2001)

This study has obtained 295 heat-resistant endospore forming bacteria (Table 1) which have gone through the screening process and the strain (HY 4-3-4), which was identified as *B. amyloliquefaciens*, was the best candidate to be used for amending the granular biofertilizer for use to produce the yardlong bean. The rigorous process to obtain this bacterium should ensure that efficacy of the granular biofertilizer shall be satisfactory when this product has been commercialized and used by the yardlong bean farmers. When used as an individual antagonist, this strain should also have potential to control other diseases of the yardlong bean (such as rust, leaf spot, damping-off, powdery mildew and even root-knot nematode) in Thailand (Schreinemachers et al., 2014). However, this requires further both laboratory and field investigations.

The fact that the number of *B. amyloliquefaciens* strain (HY 4-3-4) remained high (Table 5) and was effective to control the disease in the pot test (Table 6) indicated that granular biofertilizer was the

appropriate carrier of this selected bacterium. In our previous research, the bacterial antagonists have been formulated based on pharmaceutical technology using chemical substances as carrier of the microbe (Pengnoo et al., 2006; Chumthong et al., 2008; Chumthong et al., 2016). Although the techniques and carriers used were proven to be effective to obtain the final products, the technology to produce these products requires sophisticated equipment and high expenditure. This research shows the possibility of using organic substances that are common in the farm as carrier of the beneficial antagonists. Should the farmers get access to the selected strain, they can produce the product on farm using an inexpensive farm equipment.

The compatibility of *B. amyloliquefaciens* strain (HY 4-3-4) and *Rhizobium* strain TAL 638 has resulted to the increased disease control efficacy and promote yardlong bean growth (Table 3, 4, 6, and 7) (Peterson et al., 1996; Srinivasan et al., 1997; Maten et al., 1999). The field test has also consolidated the efficacy of the granular biofertilizer amended with *B. amyloliquefaciens* strain (HY 4-3-4) in the pot test (Table 7). The study by Ahmad et al. (2011) also showed that co-inoculation with *Rhizobium* and *Bacillus* strains significantly increased the nitrogen content of the shoot of lablab bean (*Lablab purpureus* L.). There is a challenge in using organic fertilizers, whether or not they will be amended with selected antagonist, in growing the crops that are commonly associated with *Rhizobium* spp. This is because there might be a negative effect of this microbe on the formation of nodules in the crops. Our strain Hy 4-3-4, which are compatible with *Rhizobium* strain TAL 638, should be tested with other strains of *Rhizobium*, that are recommended to use in growing other leguminous crops, so that this bacterium can be utilized for growing other crops.

Although chemical fungicides have been tested to control the stem and root rot of the yardlong bean (Kukulevithana et al., 2023), its widespread use may result to farmer's exposure to toxic chemicals and environmental pollution (Dall'Agnol et al., 2021; Edward, 2021). The efficacy of chemical fungicide to control diseases may also be short-lived which may make it to be unsuitable for use to control soil-borne diseases (Juroszek et al., 2022). On the contrary, the biological control using granular biofertilizer amended with the strain 4-3-4 in the soil should improve soil activity, mitigating the growth and spread of the pathogen over time (Luo et al., 2022). This should also help the farmers to reduce costs of labour and control agents because it will eliminate the need for frequent applications (Sunslow & Schroth, 1982; Windels & Kommedahl, 1982).

Our study shows that biological based method using granular biofertilizer amended the strain 4-3-4 is an appropriate option to use in growing the yardlong bean. Knowledge from this study should be transferred to the agricultural officers to promote its use among the farmers who have grown the yardlong bean nationwide. The granular biofertilizer amended with *B. amyloliquefaciens* strain (HY 4-3-4) is the appropriate tool for growing yardlong beans because it is compliance with GAP and organic production systems (Nokkoul et al., 2011; National Bureau of Agricultural Commodity and Food Standards, 2018).

Conclusion

B. amyloliquefaciens (HY 4-3-4) was selected to amend the granular biofertilizer. The population of this bacterium remained high (> 10.00 Log. number/g), after 16 months storage. In the pot and field tests, biofertilizer amended with *B. amyloliquefaciens* (HY 4-3-4) was as effective as mancozed to control the disease. The results of the study will support GAP and organic production of the yardlong bean in Thailand.

Declarations

Conflicts of interest

The authors have no conflicts of interest to declare.

Author contributions

Amornrat Chumthong: Conceptualization, Methodology, Data curation, Supervision, Formal analysis, Investigation, and Visualization. Writing: Initial draft, Review, and Editing. Pongsak Mansuriwong: Data collection, Formal analysis, Investigation, Writing evaluation, and Editing. Paranee Sawangsri: Investigation, Data curation; Writing review, and Editing. Mana Kanjanamaneesathian: Writing-review, and Editing.

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Data availability

Data are available upon request.

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Figures

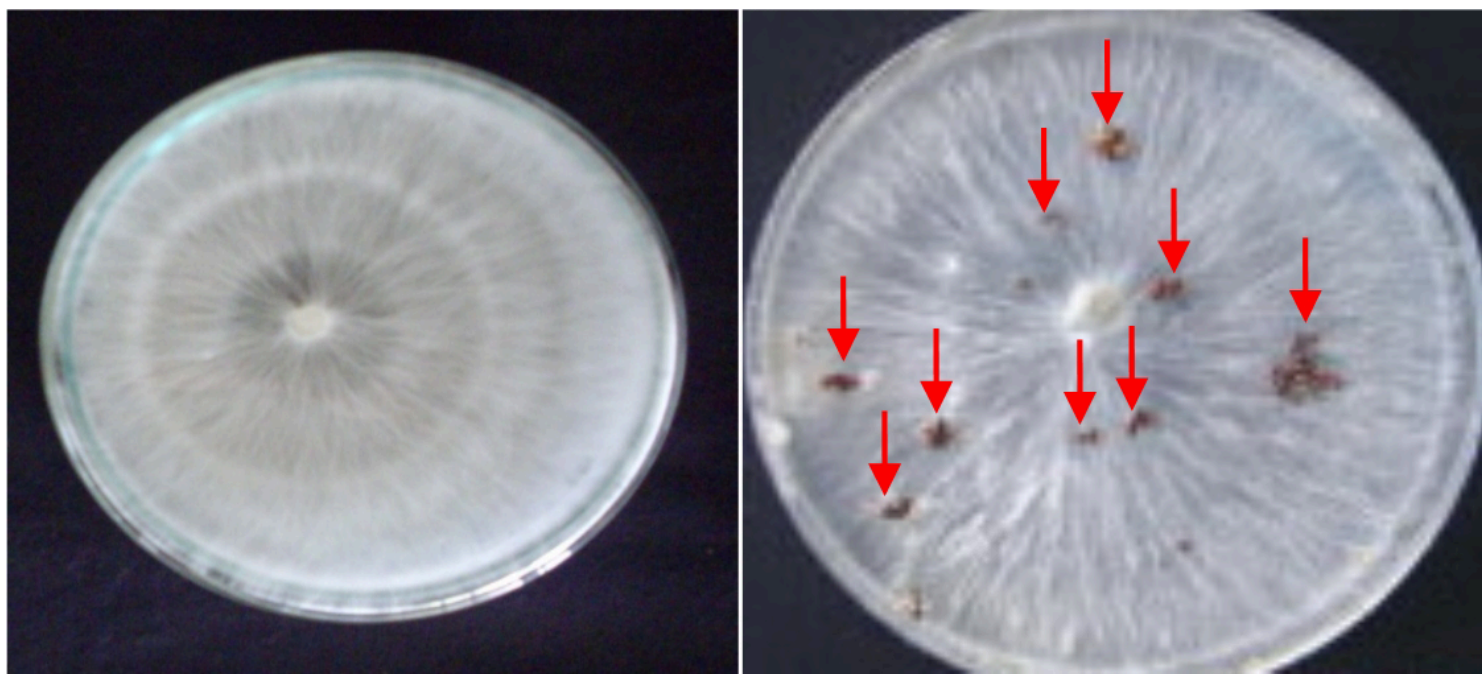


Figure 1

The hyphal growth of *Sclerotium rolfii* after incubated for 4 days on PDA medium (left), and the hyphal growth and sclerotium characteristics (arrow) of *S. rolfii* after incubated for 7 days on PDA medium (right)



Figure 2

Clear zone between *Bacillus amyloliquefaciens* (HY 4-3-4) and *Sclerotium rolfsii* (left) compared with control (right)



Figure 3

The growth of yardlong bean in only somasegaran solution (left), and with inoculation of *Rhizobium* strains TAL 638 and *Bacillus amyloliquefaciens* (HY 4-3-4) (right)