PAENIBACILLUS POLYMYXA AS POTENTIAL BIOLOGICAL CONTROL AGENT ISOLATED FROM VEGETABLES GROWN HYDROPONICALLY IN THAILAND

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ABSTRACT

The survey was conducted to collect root samples from the hydroponic vegetable farms as sources of potential bacterial antagonists for controlling root rot disease caused by *Pythium helicoides*. The root samples (in the bottles containing sterile water which had been incubated in the water bath at 84˚C for 20 minutes) were plated onto potato dextrose agar (PDA) for the isolation of the potential bacterial antagonists. There were 441 isolates of bacteria isolated from 129 root samples, in which 20 isolates showed significant inhibitory effect to the mycelial growth of *P. helicoides*. Both the sterile (at 121˚C, 15 psi for 20 minutes) and the non-sterile culture supernatant of these 20 isolates were tested further for the inhibition of mycelial growth of *P. helicoides* on the PDA. The capacity of these 20 bacteria was also tested for their capacity to inhibit the mycelial growth in potato dextrose broth (PDB). *Paenibacillus polymyxa* (isolated from *Brassica chinensis*), *P. polymyxa* (isolates from *Ipomoea aquatic*) and *P. polymyxa* (isolated from *B. alboglabra*) were selected as potential bacterial antagonists based on their capacity to produce heat stable toxic substance against the pathogen. *P. polymyxa* (isolated from *B. chinensis*), however, was also detected to produce IAA, a plant growth promoting hormone.

KEYWORDS: Plant Diseases in Hydroponics, *Pythium helicoides*

1. Introduction

Hydroponic systems have been identified as one of the intensive system for producing vegetables that is efficient in the use of production resources (e.g. water, fertilizers, and

land) with fewer pests [1]. Nevertheless, significant yield reduction in the absence of visible root or foliar symptom caused by fungal pathogen has been reported in lettuce grown hydroponically as a result of infection by *Pythium dissotocum* [2]. Yield loss in vegetables grown hydroponically can also be incurred by *P. aphanidermatum* and *P. myriotylum* [3] and insect pests [4].

Biological control of vegetable diseases grown hydroponically has been investigated in developed countries [5-9] because chemical fungicide application in hydroponic vegetables will cause the residue issue on the produces, making them unacceptable by the consumers. Both endophytic and epiphytic bacteria, particularly *Bacillus* spp. and *Paenibacillus* spp., from various resources have been reported to be effective against root rot diseases caused by *Pythium* spp. [10-13]. Epiphytic bacterium, *B. velezensis* isolated from the surface of the root of *Lactuca sativa var. crispa*, was reported to be effective in suppressing root rot disease caused by *Pythium* sp. and promoting growth of *Lactuca sativa* [14].

Products of *Bacillus subtilis* and *Trichoderma harzianum* have been commercialized and recommended for use to control diseases in vegetables grown hydroponically, particularly for root rot disease caused by *Pythium* spp. Control efficacy of these products is varied as they have been used by the growers in the paradigm of using chemical fungicide for disease control in hydroponic production system, overlooking the intrinsic characteristics of the biological control products. Furthermore, control efficacy can be improved if new isolates of the antagonists, selected from the niches where they will be applied, are isolated, screened and identified.

This research aims to (1) isolate potential bacterial antagonists, particularly the *Bacillus* spp., from root of hydroponic vegetables, (2) screen these isolated bacterial antagonists to detect the effective isolates of the bacterial antagonists and (3) identify the effective isolates to the specific level.

2. Materials and Methods

2.1 Fungal pathogen culture

Pythium helicoides was isolated from roots of green oak (*Lactuca sativa* var. *crispa*) with root rot symptom. The pathogen was cultured on PDA slant in the test tubes and these tubes were stored in the refrigerator at 4°C in the laboratory at the Faculty of Animal Science and

Agricultural Technology, Silpakorn University, Thailand. The root rot disease samples were collected from hydroponic farm using the dynamic root floating technique (DRFT) to produce the vegetables in Bangkok. This pathogen was used in the following antagonism tests because it showed high virulence to *L. sativa* var. *crispa* (such as butter head, green oak and red coral).

2.2 Sites of the hydroponic vegetable farms

The root samples of the hydroponic vegetables were collected from five provinces such as Bangkok, Ayutthaya, Chon Buri, Nonthaburi, and Phetchaburi. The systems which were used to grow the hydroponic vegetables in this survey included nutrient film technique (NFT) and dynamic root floating technique (DRFT) (Table 1). Root samples of the plant species (in Table 1) were used to isolate the potential bacterial antagonists.

Table 1 Sites and systems where roots of the hydroponic vegetables were surveyed and collected

Provinces	Hydroponic systems	Plant species
Bangkok	NFT	*Lactuca sativa* var. *crispa*
Ayutthaya	DRFT	*Brassica alboglabra*
Chon Buri	NFT/DRFT	*L. sativa* var. *crispa*
Nonthaburi	NFT	*B. pekinensis* *L. sativa* var. *capitata* *L. sativa* var. *longifolia*
Phetchaburi	DRFT	*B. chinensis* *Ipomoea aquatica* *L. sativa* var. *longifolia* *L. sativa* var. *crispa*

2.3 Isolation of epiphytic bacteria antagonists

Potential bacterial antagonists were isolated using tissue transplanting technique. The roots of the collected samples in the bottles containing sterile water were bought to the

laboratory and they were incubated in the water bath at 80˚C for 20 minutes. This practice was carried out to obtain the bacterium which produced heat-resistant endospores [15]. After the incubation, the roots from each sample were aseptically cut into small pieces (approximately 1 mm. in length). Five pieces of the root samples from each bottle were used for the isolation of the potential bacterial antagonists on the potato dextrose agar (PDA) plates, with one piece of root sample per plate [15-16]. These plates were incubated on the laboratory bench at room temperature (25-32°C) for 48 hours. The bacterium was transferred to the PDA slant for further tests [15, 16].

2.4 Antagonism tests on the agar medium

In the first round of the antagonism test, 441 isolates of bacteria isolated from 129 root samples were tested against *P. helicoides* using dual culture technique on the PDA. Each bacterium was streaked on one end of the plate and the agar plug of the *P. helicoides* was placed on the other end. This plate was incubated on the laboratory bench at room temperature (25-32°C). The inhibition of the mycelial growth of *P. helicoides* was assessed after incubation for 15 days. Clear zone between the colony of the bacterium and the fungus was measured and the bacterium with high inhibitory capacity to the fungus was selected for further tests [15].

In the second round of the antagonism test, both non-sterile and sterile culture filtrates of the selected bacterium were tested against the fungus on the PDA in the protocol as described by Pengnoo et al. [16]. Each selected bacterial growing on nutrient agar (NA) for 24 hours was transferred to sterile PDB (100 mL) in Erlenmeyer flask and incubated in the horizontal shaker (150 rpm) at room temperature for 24 hours. After 24 hours, each bacterial culture (15 mL) in the test tubes was centrifuged at 3,000 rpm for 30 minutes after which the supernatant was sieved with a sterile filter paper (0.45 μ) to get rid of the bacterial cells. This filtered supernatant was divided into two parts; the first part was sterile with autoclave at 121°C for 20 minutes at 15 psi and the second part was stored as it was (without sterilization) [16].

Either non-sterile or sterile culture filtrate of each selected bacterium was mixed with PDA at the 1:1 proportion (v/v), after which the agar plug of the *P. helicoides*, which had been cultured on PDA for four days, was placed onto the centre of the plate. The plate was incubated on the laboratory bench at room temperature (25-32°C) for four days, after which

the percentage of mycelial inhibition was calculated in Equation (1) as described by Gamliel et al. [17].

$$\% \text{ mycelial inhibition} = 100 - [(r2/R2) \times 100] \quad (1)$$

In equation (1), r is the colony radius of *P. helicoides* on PDA incorporated with either sterile or non-sterlile filtrate of the bacterium and R is colony radius of *P. helicodes* on PDA incorporated with sterile water. The experiment was done with four replications in the completely randomized design (CRD). Data were analysed using program for statistical analysis R (R-language and environment for statistical computing and graphics). Means were compared using Duncan's Multiple Range Test (P=0.01).

2.5 Identification of the selected bacterial antagonists and determination of IAA production

Bacteria that showed mycelial inhibitory capacity were identified by amplifying partial sequences of the 16S rRNA gene [18]. The selected bacteria were evaluated for their capacity to produce indole-3-acetic acid (IAA). IAA equivalents production by the bacterial isolates was assayed using the qualitative method. All isolates were plated onto nutrient agar medium amended with L-TRP, overlaid with a cellulose membrane and incubated at 30°C for 48 hours. Bacteria producing IAA were identified by the formation of a characteristic red halo within the membrane immediately surrounding the colony [19].

3. Results and Discussion

3.1 Preliminary antagonism test on PDA using dual culture technique

There were 441 isolates of bacteria isolated from 129 root samples, in which 72 isolates showed inhibitory effect to the mycelial growth of *P. helicoides* in the first round of antagonism test using dual culture technique. Among these 72 isolates, 20 isolates showed inhibitory effect to the fungus, with the clear zone between the tested bacteria and the fungus ranging from 3 to 7 mm [Figure 1(A) and (B)].

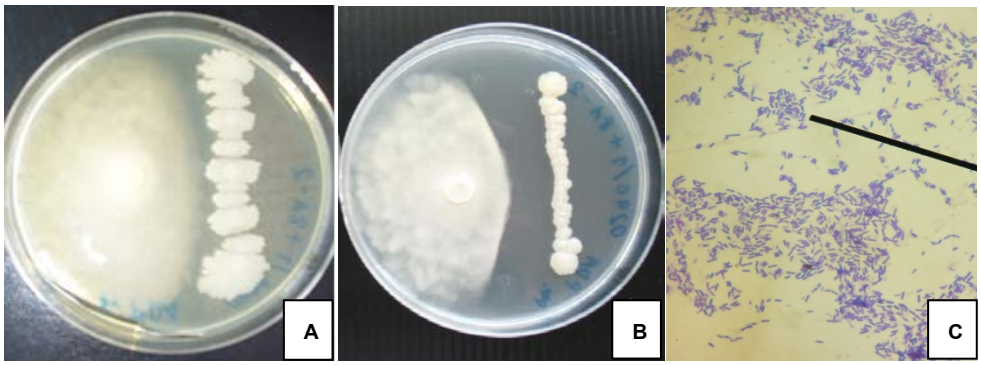

Figure 1 **(A) Clear zone created by *Bacillus velezensis* (B) by *Paenibacillus polymyxa* (isolate 1) in inhibiting *Pythium helicoides*, (C) Violet stained Gram-positive of *Paenibacillus polymyxa* (isolate 4) cells.**

3.2 Antagonism tests using sterile and non-sterile culture filtrate

There were five isolates of the tested bacteria which showed high antagonistic activity against *P. helicoides*, based on the tests using either sterile or non-sterile culture filtrate incorporated into PDA (Table 2).

3.3 Identification of the selected bacterial antagonists and determination of IAA production

The species of the selected bacterial antagonists and their capacity to produce IAA qualitatively were shown (Table 2).

Various ecosystems, as a potential habitat of the undiscovered *Bacillus* spp. which possesses the useful substances for agricultural application, include coral reefs, mangrove and coastal vegetation, tropical evergreen forests, wetlands, coastal marshes and swamps, and the specific niches in an agro-ecosystem (such as agricultural soil, infertile soil, seed and plant tissue) [11].

B. megaterium, isolated from soil in paddy rice field in Thailand, and its products, was effective to control major rice diseases in Thailand [20]. *B. velezensis,* the epiphytic bacterium isolated from root of *L. sativa var. crispa* grown hydroponically, had potential to promote the growth of *L. sativa* (var. Red Coral) [14].

Table 2 Species of selected bacterial antagonists, their inhibitory capacity to mycelial growth of *Pythium helicoides* and IAA production

Treatments with selected bacteria	%inhibition		IAA	Sources of selected bacteria
	Sterile	Non-sterile		
Paenibacillus polymyxa 1	15.0B*	100.0A**	negative	*Brassica alboglabra*
P. polymyxa 7	0.0B	100.0A	negative	*B. alboglabra*
Bacillus amyloliquefaciens 3	0.0B	25.0B	negative	*Lactuca sativa var. capitata*
P. polymyxa 4	75.5A	100.0A	positive	*B. chinensis*
P. polymyxa 2	67.0A	100.0A	negative	*Ipomoea aquatica*
B. velezensis 1	0.0B	0.0B	positive	*L. sativa var. crispa*
Only *Pythium helicoides*	0.0B	0.0B	-	-

*, ** Means in each column followed by the same letter are not significantly different at P=0.01.

Four isolates of *P. polymyxa* (isolates 1, 7, 4 and 2), isolated from the root samples of the vegetables grown hydroponically, had high inhibitory effect to the mycelial growth of *P. helicoides* (Table 2) when the culture filtrates (either sterile or non-sterile) were used for the antagonism tests. This may be because the substances which were highly antagonistic to the fungal pathogen were excreted from the bacterial cells and showed their potency in fungal inhibition when they were amended in the medium. The four isolates of *P. polymyxa*, for example *P. polymyxa* 1, did also show obvious antagonism against the pathogen when they were screened against the pathogen in the dual culture [Figure 1 (B)]. It is possible that *P. polymyxa* (isolates 1, 4 and 2) may possess multiple mechanisms in pathogen suppression as indicated by the fact that both sterile and non-sterile culture filtrates from both isolates are effective in inhibiting the pathogen (Table 2).

On the other hand, *Bacillus velezensis*, which showed some effect in inhibiting the pathogen [Figure 1 (A)] in the normal dual culture antagonism test, did not secret the substances which were highly toxic to the pathogen (Table 2), making it to be a poor candidate for selection for use in further development. Nevertheless, this bacterium may

possess other beneficial mechanisms which may be contributory to disease suppression and plant growth promotion [14].

The fact that both sterile and non-sterile culture filtrates of this bacterium (isolates 4 and 2) were highly antagonistic to the mycelial growth of the fungus may indicate that both heat-labile and heat-stable substances may contribute to the growth inhibition, whilst only heat-labile substances produced by *P. polymyxa* (isolate 7) may be the substances which inhibit the mycelial growth. Further research is required to determine the nature of the substances which are responsible for the inhibition of mycelial growth of *H. helicoides* [21].

With respect to the effect to plants, other *Paenibacillus* species were reported to promote crop growth directly via biological nitrogen fixation, phosphate solubilization, production of the phytohormone indole-3-acetic acid (IAA), and release of siderophores that enable iron acquisition. These bacteria also protect plants from infection by various fungal plant pathogens [13]. Yang et al. [10] reported the efficacy of the strain PKB1 of *P. polymyxa* to control disease caused by *Pythium* sp. in vegetables grown hydroponically. *P. polymyxa* (isolates 1, 7, 4 and 2), isolated from the hydroponic system and screened for their antagonistic activity in the laboratory, should show good efficacy for control root rot disease caused by *Pythium* spp. This, however, requires testing to determine their efficacy with vegetables grown hydroponically.

P. polymyxa (isolate 4) [Figure 1 (C)], isolated from *Brassica chinensis*, is the best candidate which should be chosen for further studies with regard to produce a formulation of this bacterium [14] because it also produces plant growth hormone, IAA (Table 2). This study consolidates the notion that hydroponic system provides an environmental niche which not only sustains the growth of plants but also gives asylum to the beneficial microorganisms which can be utilized for crop production.

4. Conclusion

Root of vegetables grown hydroponically is the habitat of beneficial bacteria such as *Bacillus* spp. and *Paenibacillus* spp. *P. polymyxa* (isolate 4) is effective in inhibiting the mycelial growth of *Pythium helicoides*. This bacterium also has potential to promote growth of the vegetables because it produces plant growth hormone, IAA.

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