Application of a suspension concentrate formulation of *Bacillus velezensis* to control root rot of hydroponically-grown vegetables

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Abstract *Bacillus velezensis* was isolated from roots of *Lactuca sativa* var. *crispa* and has been shown to be inhibitory of mycelial growth of *Pythium* spp. root rot pathogens. The bacterium was prepared in a suspension concentrate formulation and tested for control of root rot and growth promotion in hydroponically-grown vegetables. Initial results indicated that the suspension concentrate was effective in controlling root rot when applied directly to seedlings of *L. sativa*. This efficacy was, however, nullified when the formulation was applied as a suspension to raise these seedlings. This formulation, when applied as a drench treatment to seedlings of *Brassica campestris* var. *chinensis*, increased growth of this vegetable. The bacterium produced indole-3- acetic acid (IAA).

Keywords biological control, host growth promotion, pathogen inhibition.

INTRODUCTION

Biological control is an option to suppress plant diseases (Cook & Baker 1983). The adoption of biological control by farmers, however, is dependent upon the availability of effective products. In developed countries, where state regulation is stringent and the customer is aware of health issues, farmers are obliged to adopt

biological products to control plant pests. This condition has led to the successful introduction of biological products to control plant diseases in the USA (Schisler et al. 2004).

In Thailand, research on the use of biological control to suppress plant pathogens has been carried out with damping-off of cucumber

(Intana 2003), leaf blight of bambara groundnut (Pengnoo et al. 2006) and sheath blight of rice (Wiwattanapatapee et al. 2004; Wiwattanapatapee et al. 2007; Chumthong et al. 2008). Some of these studies have led to the commercialisation of the biological control agent, *Trichoderma harzianum* (Intana 2003) for control of soil-borne plant pathogens. These studies have identified effective antagonists, evaluated fresh cultures in laboratory and greenhouse trials, and transformed the antagonists into practical formulations for control of leaf and root pathogens.

Crops grown hydroponically are affected by root rot diseases caused by *Aphanomyces* sp. and *Pythium* spp., with these pathogens adapting to aquatic environments and causing severe crop losses. There are differences between the environmental regimes for above ground or soil diseases and those for diseases that occur in hydroponic situations, which means that different formulations are required for effective control of root rots in hydroponically-grown crops (Kanjnamaneesathian et al. 2013). Although there are numerous farms growing vegetables hydroponically throughout Thailand, there is no previous research on formulating bacterial antagonists to combat root diseases in these crops. Large farms grow vegetables (mainly *Lactuca sativa*) for hotel and restaurant markets. Smaller farms grow “common” vegetables (such as *Brassica campestris*) for customers prepared to pay extra for pesticide-free produce.

This present research aimed to: (1) test the efficacy of suspension concentrates of the bacterium *Bacillus velezensis* against root rot of *L. sativa* caused by *P. aphanidermatum* in the laboratory; and (2) evaluate plant growth promotion of *B. campestris* var. *chinensis* by the bacterium in the dynamic root floating technique (DRFT) hydroponic system.

MATERIALS AND METHODS

Isolation and identification of antagonistic bacteria

Roots of vegetables [Pak Choi cabbage (*B. chinensis*), Chinese cabbage (*B. pekinensis*),

swamp cabbage (*Ipomoea aquatica*), Chinese kale (*B. alboglabra*), cos lettuce (*L. sativa* var. *longifolia*), butterhead lettuce (*L. sativa* var. *capitata*) and red coral lettuce (*L. sativa* var. *crispa*)] were collected from farms growing vegetables hydroponically in five provinces (Bangkok, Nonthaburi, Ayutthaya, Chonburi and Phetchaburi). Root samples were put in sterile water in bottles and subjected to 80°C hot water treatment for 20 min in a waterbath. An aliquot from each of these bottles was streaked onto potato dextrose agar (PDA) to isolate the endospore-forming bacteria. Single colonies of bacteria were streaked onto PDA slants in test tubes and stored for testing of capacity to inhibit mycelial growth of *P. helicoides* and *P. aphanidermatum* on PDA (Kanjnamaneesathian et al. 2010). Bacteria that showed mycelial inhibitory capacity were identified by amplifying partial sequences of the 16S rRNA gene (Kanjnamaneesathian et al. 2010). The selected bacteria were evaluated for their capacity to produce indole-3- acetic acid (IAA), using the method as described by Abd El-Azeem et al. (2007). Bacterial cells were also subjected to Gram staining and endospores were measured micrometrically.

Formulation preparation

Bacillus velezensis was chosen for the formulation study and the suspension concentrate of this bacterium was used in all the following tests. An optimum suspension concentrate of *B. velezensis* contained 10^{12} CFU/ml endospores (400 ml), sterile water (500 ml) and 5% xanthan gum (w/v) (100 ml). These components were mixed using a magnetic stirrer until a uniform suspension was obtained. A suspension concentrate, without endospores of the bacterium, was also prepared and was used in the efficacy test as an experimental control. This suspension concentrate was tested both for efficacy in suppressing infection of *L. sativa* seedlings (in two laboratory tests), and for promoting seedling growth of *B. campestris* var. *chinensis* in one glasshouse test using the DRFT plant growth system.

Laboratory test with *L. sativa*

In the first test, seeds of *L. sativa* were germinated on water-soaked sponges in plastic micro-pot trays (with bottom openings to allow root growth) for 14 days. After germination, these seedlings were removed from the trays and placed on top of water-soaked sponges in new trays, sandwiching agar plugs of mycelia of *P. aphanidermatum*. These trays were then drenched with 500 ml of either tap water (nil experimental control), bacterial suspension concentrate or fresh cells. There were four treatments (with four replicates, each with four seedlings). The treatments were: (1) seedlings receiving tap water (nil experimental control), (2) seedlings receiving 1% formulation (v/v) (experimental control), (3) seedlings receiving 1% suspension concentrate (10^{12} CFU/ml) (v/v) and (4) seedlings receiving 1% fresh cells of *B. velezensis* (10^{14} CFU/ml) (v/v). Sponges containing growing seedlings were placed on top of agar blocks of mycelia of *P. aphanidermatum*, and numbers of the surviving seedlings were assessed 15 and 30 days after inoculation.

In the second test, seedlings of *L. sativa* were prepared as described above, except that they were allowed to grow in the trays until their roots emerged from the bottom openings in the trays. The trays were then placed in plastic containers (dimensions 18 × 27 × 10 cm) containing tap water (2 litres) mixed with 1 litre of either (1) tap water as a nil experimental control, (2) 1% formulation (experimental control), (3) 1% suspension concentrate (10^{12} CFU/ml) or (4) 1% fresh cells of *B. velezensis* (10^{14} CFU/ml). Six agar plugs of mycelia of *P. aphanidermatum* were then added to each container. Numbers of the surviving plants were assessed at 30 days after inoculation. Twelve plants were also randomly selected from each treatment at the end of the experiment, and ten pieces of root tip from each plant were aseptically excised and plated onto PDA. The numbers of root tips colonised with the fungus were counted after 1 day of incubation, and percentage of colonised root tips was calculated.

Glasshouse test in the DRFT system with *B. campestris* var. *chinensis*

Determination of *B. campestris* var. *chinensis* growth promotion was carried out in the DRFT system at Phetchaburi College of Agriculture and Technology, Thailand. Twenty-five seedlings growing for 14 days on the platform on the DRFT system were drenched with 5 ml of either 1% formulation (v/v) (experimental control), 1% suspension concentrate (10^{12} CFU/ml) (v/v) or 1% fresh cells of *B. velezensis* (10^{14} CFU/ml) (v/v), while 25 non-treated seedlings served as nil experimental controls. Six seedlings growing in the centre of the platform were randomly sampled 14 days after the treatments were applied. Lengths of shoots and roots were measured, and fresh and dry weight of shoots and roots were determined.

Statistical analysis

Data collected were subjected to statistical analysis using the SAS Software Program (Version 9.1). Means ($\pm$ SE) of the length of endospores, percent surviving seedlings of *L. sativa*, percent root tips of *L. sativa* colonised by the pathogen, shoot and root lengths, and fresh and dry weights of *B. campestris* var. *chinensis* plants were compared using a Duncan Multiple Range Test.

RESULTS

Isolation and identification of antagonistic bacteria

In total, 1,182 isolates of bacteria were obtained from roots of hydroponically grown vegetables. Of these, 441 isolates were tested for inhibition of mycelial growth of *P. helicoides* and *P. aphanidermatum*. Seventy-two isolates inhibited mycelial growth of these two fungi on PDA. Among these 72 isolates, six isolates of endospore-forming bacilli gave greater inhibition than other isolates and were chosen for the formulation study. These isolates were also selected based on other attributes, such as the size of their endospores and capacity to produce IAA (Table 1), but *B. velezensis* was chosen for the formulation study and other tests.

Table 1 Species of six endospore-forming bacilli, with respective spore shapes and mean sizes, selected for the formulation study, n=300. Means followed by the same letter are not significantly different (P = 0.01).

Isolates	Identification	Spore shape	Spore length (µm)	IAA production ¹
1. 029 C/1	<i>Paenibacillus polymyxa</i>	coccus	1.07 e	-
2. 029 C/7	<i>P. polymyxa</i>	coccus	1.57 cd	-
3. 097 A/3	<i>Bacillus amyloliquefaciens</i>	bacillus	1.22 de	-
4. 121 C/4	<i>P. polymyxa</i>	bacillus	1.80 c	+
5. 123 B/2	<i>P. polymyxa</i>	coccus	2.26 b	-
6. 129 C/1	<i>B. velezensis</i>	bacillus	2.69 a	+

¹- or + indicates either a negative or positive IAA production respectively.

Formulation preparation

The optimum suspension concentrate formulation was selected because it had good dispersion characteristics, compared with other formulations in which the ingredients may quickly form sediments. No sedimentation occurred in the optimum formulation during a 4-week storage period at room temperature (26-32°C).

Laboratory test with *L. sativa*

The suspension concentrate and fresh bacterial cell treatments suppressed root rot, protecting lettuce seedlings and maintaining their viability, while seedlings in the nil treatment succumbed to root rot at 30 days after inoculation (Table 2).

After inoculating the pathogen, seedlings had severe root rot symptoms and the whole root had a dark-brown colour. Leaves of the seedlings became desiccated, regardless of the treatments applied to the plastic containers. Nevertheless, the suspension concentrate reduced the percentage of root tips that were colonised by the inoculated pathogen (Table 3).

Glasshouse test in the DRFT system with *B. campestris* var. *chinensis*

Drenching the suspension concentrate or fresh bacterial cells onto *B. campestris* var. *chinensis* increased root and shoot lengths. Fresh cells of the bacterium increased root fresh weight, while the suspension concentrate increased fresh weight of the shoots. None of the treatments affected dry weights of the root, while the formulation alone (experimental control) reduced shoot dry weights (Table 4).

DISCUSSION

Roots of hydroponically-grown vegetables were good sources of antagonistic bacteria, particularly in the genera *Bacillus* and *Paenibacillus*. *Bacillus velezensis* (isolate No. 129/1) was isolated from roots of *L. sativa* var. *crispa*, grown in the DRFT system (Table 1). The growth promoting capability of this bacterium (Table 4) would mitigate the effect of disease on plants, providing another advantageous characteristic to its inhibitory effect on the pathogen. Furthermore,

Table 2 Mean (±SE) of percent surviving seedlings of *Lactuca sativa* after different *Bacillus velezensis* treatments had been applied and inoculating with the pathogen *Pythium aphanidermatum*, n=16. Means in each column followed by the same letter are not significantly different (P= 0.01).

Treatment	15 days	30 days
Nil (control)	75.06 ± 4.80 b	8.34 ± 4.81 b
1% formulation	95.83 ± 4.16 a	83.34 ± 9.62 a
1% bacterial suspension concentrate	100.00 ± 0.00 a	91.67 ± 4.81 a
1% fresh bacterial cells	100.00 ± 0.00 a	87.50 ± 7.97 a

Table 3 Mean ($\pm$ SE) of percent root tips of *Lactuca sativa* colonised by the pathogen *Pythium aphanidermatum* at 30 days after treatment with the bacterium *Bacillus velezensis*, n = 12. Means in each column followed by the same letter are not significantly different (P = 0.01).

Treatment	% colonized root tips
Nil (control)	96 $\pm$ 4.0 a
1% formulation	88 $\pm$ 7.4 a
1% bacterial suspension concentrate	70 $\pm$ 7.4 b
1% fresh bacterial cells	81 $\pm$ 8.1 ab

the relatively large endospores of this species may extend its survival. *Bacillus velezensis* had been reported to produce a surfactant substance, which may inhibit *Pythium* spp. (Ruiz-Garcia et al. 2005). Further study would be worthwhile to determine if the ability of isolate No. 129/1 to produce surfactant is a mechanism for antagonistic capability against the root rot pathogens.

The fresh cells of isolates No. 129/1, screened and selected on the basis of mycelial inhibition, and the suspension concentrate formulation, were effective in suppressing root rot, protecting lettuce seedlings and maintaining their viability (Table 2). The use of mycelial plugs of the pathogen as inoculum during the test may have contributed to the effectiveness of these treatments. The efficacy recorded for the formulation alone treatment cannot be explained. However, xanthan gum may have fungicidal activity, or it may act as an elicitor to induced resistance against disease, as reported by Antoniazzi et al. (2008) for disease of barley caused by *Bipolaris sorokiniana*.

The effects of the isolate No. 129/1 in controlling root rot were nullified when the formulations were applied as suspensions in the boxes used to raise seedlings. It is possible that the added formulation was not effective in preventing root infection by pathogen zoospores and subsequent colonisation. Severe root rot symptoms occurred followed by desiccation of all plants. However, the suspension concentrate reduced proportions of root tips that were colonised by the pathogen (Table 3). This effect may be because the suspension concentrate reduced zoospore attachment and infection of roots. Nevertheless, this observation should be confirmed with further laboratory investigation.

Drenching the suspension concentrate onto *B. campestris* var. *chinensis* increased fresh weights of the shoots (Table 4). The growth enhancement may be because isolate No. 129/1 is capable of producing IAA (Table 1). This bacterium and the suspension concentrate could be used to protect the seedlings from infection by *P. aphanidermatum* and to promote seedling growth, particularly when seedlings of *L. sativa*

Table 4 Mean ($\pm$ SE) shoot and root lengths, fresh and dry weights of *Brassica campestris* var. *chinensis* plants after being treated with the bacterium *Bacillus velezensis*, n=24. Means in each column followed by the same letter are not significantly different at P = 0.05.

Treatment	Length (cm)		Fresh weight (g)		Dry weight (g)	
	Root	Shoot	Root	Shoot	Root	Shoot
Nil (control)	27.1 $\pm$ 1.3b	33.6 $\pm$ 1.3b	2.3 $\pm$ 0.2b	21.0 $\pm$ 4.8b	0.4 $\pm$ 0.0	1.0 $\pm$ 0.1a
1% formulation	35.4 $\pm$ 4.3b	34.7 $\pm$ 1.0b	3.0 $\pm$ 0.4b	25.4 $\pm$ 3.5ab	0.4 $\pm$ 0.0	0.7 $\pm$ 0.1b
1% bacterial suspension concentrate	48.6 $\pm$ 5.5a	37.9 $\pm$ 0.9a	3.1 $\pm$ 0.2b	34.3 $\pm$ 3.8a	0.4 $\pm$ 0.0	1.3 $\pm$ 0.1a
1% fresh bacterial cells	45.8 $\pm$ 3.4a	38.3 $\pm$ 1.1a	4.2 $\pm$ 0.7a	30.1 $\pm$ 2.9ab	0.4 $\pm$ 0.0	1.2 $\pm$ 0.1a

or *B. campestris* var. *chinensis* are raised in the seed germination boxes before transplanting to the DRFT system. The effect of the suspension concentrate to enhance growth of these and other vegetables should also be investigated, as other mechanisms, such as phosphate solubilisation and siderophore production, may contribute to the promotion of growth (Abd El-Azeem et al. 2007).

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