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Influence of organic amendment and *Bacillus subtilis* on mineral nutrient uptake of asparagus bean in two field soils

Minh Thanh Ha[1], Yuh-Ming Huang[2], and Jenn-Wen Huang[1, 3]

[1] Department of Plant Pathology, National Chung Hsing University, Taichung 402, Taiwan

[2] Department of Soil and Environmental Sciences, National Chung Hsing University, Taichung 402, Taiwan

[3] Corresponding author, E-mail: jwhuang@dragon.nchu.edu.tw; Fax: +886-4-2285-1676

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ABSTRACT

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Greenhouse experiments were carried out to evaluate efficacy of soil amendment with shrimp and crab shell powder (SCSP) and *Bacillus subtilis* strain PMB-034 on control of Fusarium wilt of asparagus bean caused by *Fusarium oxysporum* f. sp. *tracheiphilum* and growth of bean plants. Results showed that amendment of soils, from Taichung or Dali, with SCSP at 0.5% (w/w) and *Bacillus subtilis* (10^8 cfu/g soil) were effective in controlling Fusarium wilt of asparagus bean and improving seedling uptake of mineral nutrients and seedling growth. Compared to untreated control, the treatment of SCSP at 0.5% and *Bacillus subtilis* PMB-034 (10^8 cfu/g soil) reduced disease severity by 50% and 62% in Taichung and Dali soils, respectively. The treatment also increased shoot dry weight by 39.3% and 57.7% in Taichung and Dali soils, respectively. Results of analyses of nutrient elements in shoots of asparagus bean showed that SCSP and *B. subtilis* PMB-034 had a stimulatory effect on nutrient uptake of asparagus bean in Taichung soil where the concentrations of macro-element nutrients including N, P, K, Ca, Mg increased by 50-67%, and micro-element nutrients including Fe, Mn, Cu, Zn increased by 57-98%. In contrast, this treatment did not cause a significant effect on nutrient uptake of asparagus beans grown in Dali soil. This study suggests that soil source is an important factor affecting uptake of mineral nutrients of asparagus bean plants grown in infested and non-infested soils with or without amendment of SCSP and *B. subtilis*.

Key words: Asparagus bean, *Bacillus subtilis*, *Fusarium oxysporum* f. sp. *tracheiphilum*, Fusarium wilt, plant nutrient uptake, organic amendment, shrimp and crab shell powder

INTRODUCTION

Macro-elements such as nitrogen, phosphorus and potassium as well as micro- elements such as iron and manganese are essential nutrients for plant growth and development. However, the widespread and heavy application of agrochemicals for crop production is known to cause negative impacts on arable soils [20, 28].

Soil microorganisms are important in agriculture as many of them are capable of promoting the circulation of

plant nutrients and reducing the need for chemical fertilizers. For instance, plant growth promoting rhizobacteria (PGPR) may be used as biofertilizer to increase N and P uptake by plants and thereby, promote growth and production of crops [6]. The mechanisms of plant growth stimulation by PGPR are mobilization of nutrients [22], stimulation of root growth by production of phytohormones [4] and antagonism against soilborne plant pathogens [14]. All of these mechanisms pre-suppose a direct contact between bacteria and the root surface and an active status of the introduced bacteria. Different PGPR including species of *Azospirillum*, *Bacillus*, *Pseudomonas*, *Enterobacter* have been used for their beneficial effects on plant growth [14, 18].

In intensively cropped tropical soil, indiscriminate use of chemical fertilizers has resulted in low organic matter content and imbalance in nutrient supply by depletion of essential micronutrients [25]. Organic amendments can improve aggregation, reduce compaction and surface crusting, increase C sequestration and nutrient availability, and enhance infiltration and water holding capacity of soil [15, 24]. One of the possible ways of enhancing productivity of acid soil is the use of alkaline fly ash and other industrial wastes in combinations with organic matter and chemical fertilizers which would act as a soil amendment and source of nutrient [25]. The addition of amendments such as fly ash, pig manure, sewage sludge, is effective in lowering the metal toxicity of soil and provides a slow release of nutrient sources such as N, P, K to support plant growth [9, 30].

Shrimp and crab processing wastes containing chitin, protein and calcium carbonate are generally pre-treated by the processes of size reduction, deproteination, and demineralization to obtain a chitin suitable for bioconservation or other use [1, 10]. Information is meager on effect of organic soil amendment and a microorganism on plant growth, nutrient uptake of asparagus bean. The objectives of this research were to evaluate the effect of soil amendment with shrimp and crab shell powder and *Bacillus subtilis* strain PMB-034 on growth and nutrient uptake of asparagus bean in two field soils.

MATERIALS AND METHODS

Soil, plant and organic material

The soils used in this study were collected from vegetable fields in counties of Taichung and Dali, Taiwan. They were sieved through a 4-mm-mesh screen, adjusted moisture to 10% (w/w) and stored in plastic containers for use in the experiments, and through a 2-mm-mesh screen for use in the analyses of nutrient components. The properties of soils are presented in Table 1. The shrimp and crab shell powder was purchased from FwuSow Industry Co., Ltd., Taichung, Taiwan. Seeds of asparagus bean (*Vigna unguiculata* (L.) ssp. *sesquipedalis* Verdc.), cultivar Pai-pi, were supplied by "Known-you Seed" Company, Kao-Hsiung, Taiwan.

Microorganisms

Bacillus subtilis strain PMB-034 was isolated from rhizosphere soil of asparagus bean plants grown in a field in Taichung. This strain was originally identified using standard biochemical methods, but its identity was further evaluated by 16S rRNA gene sequence analysis. Stock culture of *B. subtilis* strain PMB-034 was preserved in distilled water and kept at room temperature ranged from 28℃ - 30℃ in the Plant Disease Management Laboratory, Department of Plant Pathology, National Chung Hsing University, Taichung, Taiwan.

Fusarium oxysporum f. sp. *tracheiphilum* PMF 05 used in this study was isolated from a diseased plant of asparagus bean grown in central Taiwan. Single spore cultures of strain PMF 05 were maintained on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) in Petri dishes (9 cm diameter) at 28℃ under 12h-photoperiod. Three agar discs (1cm diameter) containing mycelial mats were removed from 5-day-old cultures and placed in a 500 ml glass flask containing 300 g of autoclaved celery pieces. After incubation at 28℃ for 7

Table 1. Chemical properties of the soil samples collected from Taichung and Dali, Taiwan

Source	Organic matter (%)	pH (1:1)	N (mg/kg)	P (mg/kg)	K (mg/kg)	Ca (mg/kg)	Mg (mg/kg)	Cu (μg/kg)	Fe (μg/kg)	Mn (μg/kg)	Zn (μg/kg)
Taichung	2.1	6.1	4.9	25.9	31.8	1015	166.0	10.6	56.4	97.5	52.3
Dali	3.7	6.8	98.7	131.0	262.0	1240	161.2	19.9	785.0	96.6	25.0

days, the infested celery pieces were thoroughly mixed with autoclaved soil. The moisture content of the soil was adjusted to 20% (v/w) by adding sterile distilled water. The soil infested with *F. oxysporum* f. sp. *tracheiphilum* was placed in plastic containers (55 cm×45 cm×15 cm, length×width×height) and kept at room temperature ranged from 28℃-30℃ for 30 days. The pathogen-infested soils were then air-dried for 10 days, placed in a refrigerator (6℃) and used as a stock inoculum for experiments. The air-dried infested soil (stock inoculum) was ground and passed through a 1.7-mm meshed screen before use. The infested soil for experiments was prepared by thoroughly mixing a stock inoculum with Taichung and Dali soils. The inoculum density of *F. oxysporum* f. sp. *tracheiphilum* in the infested soil was adjusted to 10^4 cfu/g soil.

Inoculation procedure and plant growth

The study of the effects of shrimp and crab shell powder and a bacterial strain on plant growth and nutrient uptake of asparagus bean was carried out in the greenhouse using Taichung and Dali soils. Seeds of asparagus bean were kept in moisture paper towel at 28℃ for 4 days for germination prior to planting. Non-infested and infested soils with *F. oxysporum* f. sp. *tracheiphilum* (10^4 cfu/g soil) were amended with shrimp and crab shell powder at rate of 0.5% (w/w), with or without *B. subtilis* strain PMB-034 (10^8 cfu/g soil), and incubated at 28℃. The pathogen-infested soil without shrimp and crab shell powder and bacterial suspension was used as a control. The amended or non-amended soils were placed in plastic pots (18.5 cm in diameter), 2 kg of soil/pot. The moisture content in each pot was adjusted to 15% (v/w) by adding sterile distilled water. After 7 days incubation, germinated seedlings were transplanted in pots, 2 seedlings/pot. The treatments were arranged in a complete randomization design with four replicates (pots) per treatment. The soil was moistened with water and maintained at 60% of moisture holding capacity (MHC). Individual plants were rated for severity of Fusarium wilt at 28 days after transplanting, using a scale of 0 to 3, where 0 = healthy plants, no visible symptoms; 1 = weakly infected plants showing vascular discoloration but no leaf yellowing; 2 = moderately infected plants showing leaf yellowing and wilt of plants; 3 = severely infected plants showing plant death. The disease severity for each replicate of each treatment was calculated by the formula: $(\Sigma S_i \times N_i) \times 100 / (3 \times N_t)$, where S_i is the severity ratings 0 to 3, N_i is the number of plants in each ratting, and N_t is the total number of rated plants.

Four weeks after transplanting, each plant was removed, separated into roots and shoots (stem and leaves) and dried at 70℃ for 48 h before determining shoot dry weight. The criteria for growth promotion were studied as shoot dry matter and nutrient contents of plants. The harvested plants were gently pulled out of soil and cut at shoot base to separate the root. The shoots were weighted, washed with tap water and then with deionized water, oven dried and weighted before pulverizing. The metal (K, Ca, Mg, Fe, Mn, Cu, and Zn) concentrations of shoots were measured by inductively coupled plasma atomic emission spectroscopy (ICP; Zobin - yvon, Vltimac, France) followed a wet digestion in HNO_3 and $HClO_4$ solutions [31] and total nitrogen concentration was measured by distillation method after plant tissues digested with H_2SO_4 and H_2O_2 [26]. Soil pH was measured in deionized water using a solid: liquid ratio of 1:1 (w/v) after 1 hour stirring to achieve equilibration [23]. Exchangeable K, Ca, and Mg were determined using 1 M ammonium acetate (pH 7.0) as exchangeable base [11]. Available phosphate was estimated by Bray No.1 method [5].

Statistical analysis

Data of disease severity, plant shoot biomass, plant nutrients collected from individual experiment were subjected to analysis of variance (ANOVA) for a complete randomization design. Means of the treatments in each experiment were compared using Duncan's multiple range tests and Fisher's least significant difference at 5% level. All statistical analyses were conducted with SAS/STAT software (SAS Institute Inc. 1989) [27].

RESULTS

Amendment of soil with shrimp and crab shell powder and *Bacillus subtilis* strain PMB-034 alone or combined was effective in reducing Fusarium wilt of asparagus bean under greenhouse conditions (Table 2). The most effective treatment were infested soil amended with shrimp and crab shell powder 0.5% (w/w) and *B.*

subtilis strain PMB-034 with disease severity of 21% and 17% in Taichung and Dali soils compared to 42% and 46%, respectively, in the control, at 4 weeks after planting.

The amendment of soil with shrimp and crab shell powder at 0.5% (w/w) and *B. subtilis* strain PMB-034 at 10^8 cfu/g soil significantly (P<0.05) increased shoot dry weight of asparagus bean seedlings grown in non-infested and infested Taichung, and in infested Dali soils compared to control. In Taichung soil, the most effective treatments for growth of asparagus bean were the soil amended with shrimp and crab shell powder without infestation of *F. oxysporum* f. sp. *tracheiphilum* PMB 05 or the soil treated with both *B. subtilis* PMB-034 and shrimp and crab shell powder with infestation of the pathogen, where the shoot dry matter increased by 35.7% and 39.3%, respectively, as compared to control (Table 3). In Dali soil, only treatment of its infested soil with *B. subtilis* PMB-034 and shrimp and crab shell powder did significantly enhance 57.6% of the shoot dry matter as compared to control. Other treatments did not cause a significant increase in shoot dry weight of asparagus bean (Table 3).

Amendment of the Taichung soil with 0.5% (w/w) shrimp and crab shell powder and *B. subtilis* PMB-034 also significantly increased macro-nutrient contents of

Table 2. Effect of shrimp and crab shell powder (SCSP) and *Bacillus subtilis* PMB-034 on disease severity of Fusarium wilt of asparagus bean caused by *Fusarium oxysporum* f. sp. *tracheiphilum* PMF 05 in the greenhouse

Treatment[1]	Disease severity (%)[2]	
	Taichung soil	Dali soil
Non-infested soil	0	0
Non-infested soil + SCSP 0.5%	0	0
Non-infested soil + PMB-034	0	0
Non-infested soil + SCSP 0.5% + PMB-034	0	0
Infested soil	42	45
Infested soil + SCSP 0.5%	25	29
Infested soil + PMB-034	29	25
Infested soil + SCSP 0.5% + PMB-034	21	17
$LSD_{0.05}$[3]	15.8	13.8

[1] The soils collected from fields in Taichung and Dali, were artificially infested or non- infested with *F. oxysporum* f. sp. *tracheiphilum* PMF 05. These soils were treated with shrimp and crab shell powder at 0.5% (w/w), with or without *B. subtilis* PMB-034, for 7 days before use for planting asparagus bean seedlings. 2 Four-day-old seedlings were transplanted into pots with

[2] seedlings per pot. Disease severity was recorded 4 weeks after transplanting, using the scale of 0 to 3: 0 = healthy plants, no visible symptoms; 1 = weakly infected plant showing vascular discoloration but no leaf yellowing; 2 = moderately infected plant showing leaf yellowing and wilt of plants; 3 = severely infected plant showing plant death. The disease severity for each replicate of each treatment was calculated by the formula: $(\Sigma S_i \times N_i) \times 100 / (3 \times N_t)$, where S_i is the severity ratings 0 to 3, Ni is the number of plants in each ratting, and Nt is the total number of rated plants.

[3] Means (n=4) within each column are separated by Fisher's least significant difference at $P = 0.05$.

Table 3. Influence of shrimp and crab shell powder (SCSP) and *Bacillus subtilis* PMB- 034 on shoot dry matter weight of asparagus bean seedlings grown in two field soils (greenhouse experiments)

Treatment	Dry weight (g/shoot)[1]	
	Taichung soil	Dali soil
Non-infested soil	0.28 a[2]	0.27 a
Non-infested soil + SCSP 0.5%	0.38 b	0.37 ab
Non-infested soil + PMB-034	0.37 b	0.38 ab
Non-infested soil + SCSP 0.5% + PMB-034	0.38 b	0.34 ab
Infested soil	0.28 a	0.26 a
Infested soil + SCSP 0.5%	0.35 ab	0.34 ab
Infested soil + PMB-034	0.33 ab	0.32 ab
Infested soil + SCSP 0.5% + PMB-034	0.39 b	0.41 b

[1] Four-day-old seedlings of asparagus bean were transplanted into pots with 2 seedlings per pot. Shoot dry matter weight was measured 4 weeks after transplanting.

[2] Means within the same column followed by the same letter are not significantly different ($P > 0.05$) according to Duncan's multiple range test.

Table 4. Influence of shrimp and crab shell powder (SCSP) and *Bacillus subtilis* PMB-034 on uptake (mg/shoot) of macro-element nutrients of asparagus bean in two field soils (greenhouse experiment)

Treatment	Taichung soil					Dali soil				
	N[1]	P	K	Ca	Mg	N	P	K	Ca	Mg
Non-infested soil	9.31	0.57	5.72	8.22	1.59	10.09	0.88	7.32	9.68	1.62
Non-infested soil + SCSP 0.5%	14.08	0.86	7.59	11.28	2.20	12.87	1.04	10.78	13.25	2.17
Non-infested soil + PMB-034	11.24	0.83	7.55	10.07	1.98	13.09	1.00	10.56	12.26	2.03
Non-infested soil + SCSP 0.5% + PMB-034	11.52	0.81	8.65	10.90	1.92	11.85	0.94	9.34	10.66	1.95
Infested soil	7.52	0.72	5.20	7.86	1.35	9.38	0.76	7.41	9.56	1.82
Infested soil + SCSP 0.5%	9.31	0.78	7.46	9.18	1.55	12.57	0.94	10.58	10.94	2.00
Infested soil + PMB-034	9.93	0.71	6.26	9.09	1.63	11.53	0.92	10.00	10.26	1.86
Infested soil + SCSP 0.5% + PMB-034	11.60	0.84	8.71	11.89	2.03	13.72	0.98	11.99	11.79	2.07
$LSD_{0.05}$[2]	3.44	0.16	1.98	2.48	0.58	ns	0.19	ns	ns	ns

[1] Four-day-old seedlings were transplanted into pots with 2 seedlings per pot. Macro-element nutrients were analyzed 4 weeks after transplanting.

[2] Means (n=4) within each column are separated by Fisher's least significant difference at $P = 0.05$. ns=no significance.

Table 5. Influence of shrimp and crab shell powder (SCSP) and *Bacillus subtilis* PMB-034 on micro-element nutrient uptake (μg/shoot) of asparagus bean in two field soils (greenhouse experiment)

Treatment	Taichung soil				Dali soil			
	Fe[1]	Mn	Cu	Zn	Fe	Mn	Cu	Zn
Non-infested soil	96.7	21.9	5.7	30.0	79.5	18.3	5.8	26.7
Non-infested soil + SCSP 0.5%	132.0	21.8	8.2	36.2	111.2	25.9	8.6	33.5
Non-infested soil + PMB-034	110.3	23.6	7.8	31.5	117.0	32.4	8.1	36.0
Non-infested soil + SCSP 0.5% + PMB-034	114.8	36.3	8.5	33.4	116.5	22.4	7.1	30.3
Infested soil	87.4	17.1	6.7	25.1	97.0	25.4	6.3	28.1
Infested soil + SCSP 0.5%	144.6	21.2	7.0	32.1	110.8	22.6	12.9	39.2
Infested soil + PMB-034	102.8	24.9	6.8	31.2	98.2	24.4	7.7	28.8
Infested soil + SCSP 0.5% + PMB-034	137.7	33.8	9.9	39.7	119.6	25.9	9.1	37.7
$LSD_{0.05}$[2]	50.1	18.5	2.4	9.9	ns	ns	5.1	ns

[1] Four-day-old seedlings were transplanted into pots with 2 seedlings per pot. Macro-element nutrients were analyzed 4 weeks after transplanting.

[2] Means (n=4) within each column are separated by Fisher's least significant difference at $P = 0.05$. ns= no significance.

shoot compared to untreated control. The combined treatment of shrimp and crab shell powder and the bacterium was more effective than the treatment of shrimp and crab shell powder or the bacterium alone in infested soil (Table 4). Contents of N, P, K, Ca and Mg in shoots of asparagus bean for the treatment of 0.5% (w/w) shrimp and crab shell powder + *B. subtilis* strain PMB-034 strain PMB-034 were significantly increased by 54, 16, 67, 51 and 50%, respectively when compared to control Taichung soil infested with the pathogen. In contrast, amendment of Dali soil with shrimp and crab shell powder and *B. subtilis* PMB-034 did not improve macro-nutrients uptake in plants grown in infested or non-infested soil, excepted for the content of P in the shoot of plants grown in infested soil which was significantly higher than the control (Table 4).

For micro-nutrient uptake, treatment of Taichung soil amended with 0.5% (w/w) shrimp and crab shell powder + *B. subtilis* strain PMB-034 and infested with the pathogen significantly ($P<0.05$) increased micro-nutrient contents in shoots of asparagus bean (Table 5). Concentrations of Fe, Mn, Cu and Zn were increased by 57, 98, 48 and 58%, respectively, for the treatment of infested soil amendment with shrimp and crab shell powder and *B. subtilis* PMB-034, when compared to the control. In contrast, amendment of Dali soil with shrimp and crab shell powder

and *B. subtilis* PMB-034 did not improve micro-nutrients uptake in plants grown in pathogen-infested or non-infested soil, excepted for the content of Cu in the shoot of plants grown in pathogen-infested soil which was significantly higher than the control (Table 5). The amendment of Taichung soil with shrimp and crab shell powder and *B. subtilis* strain PMB-034 had greater stimulatory effects on plant growth and assimilation of nutrients, compared to the amendment of Dali soil.

DISCUSSION

This study reveals that amendment of soil with shrimp and crab shell powder at 0.5% (w/w) and *Bacillus subtilis* strain PMB-034 is effective in reducing severity of Fusarium wilt of asparagus bean and promoting plant growth of this crop. Benhamou and Theriault [2] reported that soil amendment with chitin and chitosan from crab shell effectively controlled Fusarium crown and root rot in tomato. The effective control of Fusarium wilt of asparagus bean observed in this study might be due to rich chitin in the shell of shrimps and crabs. Previous study indicated that amendment of soil with shrimp and crab shell powder could enhance proliferation of microbial population and microbial activity [12]. Chen *et al.* [7] indicated that the quality of organic materials affected variation of microbial respiration dehydrogenase activity and cellulase activity in the amended soil. It is suggested that shrimp and crab shell powder may promote the breakdown and mineralization of soil organic materials by inhibiting or stimulating particular components of the microbial community selectively. Biochemical activities of selected microbial populations and communities of other soil microorganisms may influence plant uptake of mineral nutrients in the soil [16].

Ha and Huang [12] reported that *B. subtilis* PMB-034 could significantly stimulate formation of root nodules of asparagus bean. Present study reveals that amendment of Taichung soil with shrimp and crab shell powder at 0.5% and *B. subtilis* PMB-034 also promoted nutrient uptake of asparagus bean. Increased nutrient uptake by plants inoculated with plant-growth promoting bacteria has been attributed to the production of plant growth regulators at the root interface, which stimulated root development and resulted in better absorption of water and nutrients from the soil [19]. One of the most commonly reported direct plant growth promotion mechanisms by PGPR is the production of plant growth substances such as auxin, gibberellins [13]. Several studies clearly showed the effect of PGPR on growth of crops under various conditions [3, 17]. Plant growth promoting bacteria have an ability to convert nutritionally important elements from unavailable to available form through biological processes [29]. However, the performance of PGPR is influenced by environmental factors such soil types, as results of present study showed that the amendment of shrimp and crab shell powder at 0.5% and *B. subtilis* PMB-034 in Taichung soil but not in Dali soil. Other reports also indicated that soil types were the dominating factor responsible for the diversity of the bacterial populations associated with plant roots [8, 21].

Present study under greenhouse conditions concludes that the combine treatment of shrimp and crab shell powder at 0.5% and *B. subtilis* PMB-034 was effective in controlling Fusarium wilt of asparagus bean and promoting growth of this crop. However, the usefulness and practicality of this application method requires further investigations under field conditions.

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摘 要

何明清[1]、黃裕銘[2]、黃振文[1, 3]. 2008. 兩種田土中添加有機物與枯草桿菌對於長豇豆植株吸收營養元素的影響. 植病會刊 17: 289-296. ([1] 台中市國立中興大學植物病理學系；[2] 土壤與環境科學系；[3] 聯絡作者，電子郵件：jwhuang@dragon.nchu.edu.tw；傳真：+886-4-2285-1676)

在溫室中評估有機添加物與微生物對於長豇豆植株生長在台中與大里土壤中吸收營養元素的影響。試驗結果發現在台中與大里土中添加 0.5% (w/w) 蝦蟹殼粉和 *Bacillus subtilis* strain PMB-034 (10^8 cfu/g soil) 分別可以有效降低 50% 與 62% 長豇豆萎凋病的罹病度；同時尚可在兩種土壤中促進長豇豆幼苗的生長。長豇豆的地上部植體分析，發現在台中土壤中添加蝦蟹殼粉與 *B. subtilis* PMB-05 可以顯著促進植株吸收營養元素；然而在大里土壤中，添加物與微生物對於促進植株吸收營養的效果卻不顯著。本研究證實在接種過病原菌的土壤中添加蝦蟹殼粉與枯草桿菌後，土壤來源是影響長豇豆植株吸收營養元素的重要因子之一。

關鍵詞：長豇豆、鐮孢菌、萎凋病、枯草桿菌、有機添加物、蝦蟹殼粉、植株營養吸收