Effects of Plant Growth-Promoting Microorganisms (PGPMs) on Plant Induction Responses and Subsequent *Spodoptera litura* Performance

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ABSTRACT Plant-growth promoting microorganisms (PGPMs) have become important microbial fertilizers lately. This soilborne microbe-plant interaction may also affect plant defense responses. However, little is known about the effect of PGPMs on induced defensive responses of various plant species. In this study, we assessed the effects of PGPMs and plant species on plant's chemistry and performance of the subsequent insect herbivore *Spodoptera litura*. Two plant species (cabbage and tomato) were inoculated with PGPMs (a fungus and bacteria). The leaves of plants treated with PGPMs were collected to feed third-instar larvae of *S. litura* and to assess the plant nutrient and defensive compounds. The results indicated that the PGPM application exerted various influences on the foliar nutrient and anti-nutrient compounds and affected the relative growth rate of *S. litura*. In summary, applying PGPMs might individually affect the induced responses and cause specific interactions. However, this study only provided partial evidence regarding this interaction effect; additional studies are required to clarify the effects of PGPMs on plant defense responses and subsequent insect herbivores.

Key Words: Plant-growth-promoting microorganisms, Trypsin inhibitor, Polyphenol oxidase, Insect performance.

I. Introduction

Virtually all crop plants suffer from infections of various kinds of pests and these damages may affect crop production.[17,22,23] Recently, to reduce the use of pesticides, alternative strategies of pest management are being developed to assist crop protection. Previous studies have revealed that induced systemic resistance (ISR) of plants might be an option for controlling insect infestation.[20,38,46,49] Particular inducers could assist plants in resisting pest invasion through physical or chemical mechanisms.[15,19,23,28,45] The evolution of such induced defensive traits can be genetically fixed [1, 3, 21]; however, the consequence of such traits might be affected by other factors.[5, 18]

Plant-growth promoting microorganisms (PGPMs) are soilborne bacteria or fungi with plant-promotion or protection activities.[2] Reports have indicated that PGPMs affect plant yield, physiology, growth, germination rate, protein, mineral, and chlorophyll contents.[2,14,24] Previous studies have indicated that PGPMs can increase the efficiency of nutrient and water uptake by roots and promote ISR.[2,9,13,16,27,40] Saravanakumar[32] indicated the plant-growth promoting rhizobacteria (PGPR) strain *Pseudomonas* can induce plant resistance. In addition, the combined effect of various fluorescent pseudomonad strains can affect the development of leaf folder (*Cnaphalocrocis medinalis*) by inducing proteinase inhibitors in rice plants. Previous studies have also revealed that cucumbers inoculated with various PGPR strains reduced the feeding of cucumber beetles.[49] The

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literature has also revealed that the relative growth rate of *Helicoverpa armigera* decreased when the cotton plants were treated with *Pseudomonas gladioli*.[29] These studies have indicated that PGPMs facilitate plants in managing insect pests by promoting plant growth and inducing resistance mechanisms.[27]

Therefore, PGPM application may play a role in inducing plant defense. Little is known, however, about the effect of PGPMs on induced defensive responses among various plant species. Even less is known about this PGPM and plant species interaction on subsequent insect herbivore performance. Therefore, in this study, we assessed the effects of PGPMs and plant species on plant's chemistry and performance of the subsequent insect herbivore *Spodoptera litura*.

II. Materials and Methods

1. Insects

The eggs of *Spodoptera litura* were collected from farmland in Taichung City, Taiwan. Then, the eggs were kept in the 250-mL plastic cup with wet cotton balls for maintain its humidity. After hatching, larvae were reared in the 250-mL plastic cup and fed by an artificial diet.[48] The plastic cup was maintained in the growth chamber (25±2˚C, 16:8 h light: dark photoperiod) and the larvae were fed until the pupa stage. Pupae were divided into males and females and then kept in the 250-mL plastic cup. After adults emerge, ten pairs of adults were placed in the plastic cylinder (21 cm height x 14.9 cm diameter) covered with tissue paper for egg laying. The adults were fed by saturated sugar solution[48] and placed in the room temperature.

2. Plants

Two plant species were used in this study including cabbage (*Brassica oleracea* L. var. *capitata* L.) and tomato (*Lycopersican esculentum* Mill. var. *cerasiforme* (Dunal) Alef.). The seeds of each plant species were first sown with the soil in 104-well plates filled with a commercial growth medium (Know-You Seed Company, Taiwan) and watered daily. Sixteen days after sowing, the seedlings were treated with PGPMs and transferred to 12.7 cm pot with a field soil. After that, the plants were placed in the greenhouse condition (25±2˚C, 16:8 h light: dark photoperiod) and watered every day. The characteristics of the soil were analyzed by the Soil Survey and Testing Center, National Chung Hsing University, Taichung, Taiwan (Table 1). Foliage of the plants was collected later for the plant growth, insect feeding, and foliar chemistry analysis 39 days after sowing.

3. PGPMs and plant species experiment.

This study was conducted to evaluate the effect of two factors, plant-growth promoting microorganisms (PGPMs) and plant species (cabbage and tomato) on plant growth, foliar chemistry and insect-feeding performance. Two PGPMs groups were used for microbial treatments: the fungus (*Meyerozyma guilliermondii*) only and the bacterial mixture. To achieve an optimal effect, mixed bacterial inoculants comprised 3 bacterial species, *Burkholderia phytofirmans*, *Rhizobium miluonense*, and *Rhizobium*

Table 1 Chemical properties of the field soil used in this study.

Property	Value
pH	6.71
EC (μS/cm)	30.9
OM (%)	0.34
Total N (g kg^{-1})	0.595
P (mg kg^{-1})	76.13404
K (mg kg^{-1})	35.75068
Ca (mg kg^{-1})	443.2
Mg (mg kg^{-1})	148.3595
Fe (mg kg^{-1})	592.1538
Mn (mg kg^{-1})	222.9418
Cu (mg kg^{-1})	6.513184
Zn (mg kg^{-1})	45.12512

lusitanum. Therefore, totally 6 different treatments were used in this experiment: (1) cabbage plants only, (2) cabbage plants treated with the fungus, (3) cabbage plants treated with the bacteria mixture, (4) tomato plants only, (5) tomato plants treated with the fungus, and (6) tomato plants treated with the bacteria mixture. The PGPMs were obtained from the Laboratory of Soil Environment Microbiology and Biochemistry, Department of Soil and Environmental Science, National Chung Hsing University. After the seeds were sown for 2 wk, the microbial suspension [fungal suspension (> 10^8 cfu mL^{-1}) and bacterial suspension (> 10^8 cfu mL^{-1})] were diluted 200 times using distilled water and subsequently poured into plastic trays. The seedlings in the 104-well plates were soaked in the microbial suspension for 15 min as the first inoculation. After 2 d, the seedlings were transplanted into 5-inch pots and treated with 50 mL of various microbial solutions, and 50 mL of water was added to plants in the pots served as control groups. The inoculations were conducted 3 times and the inoculants were diluted 100 times using distilled water in the second and third inoculations. The leaves of these plants were collected for analyzing plant growth, insect feeding, and foliar chemistry at 39 days after sowing.

4. Foliar Chemistry

We analyzed the foliar protein content, polyphenol oxidase (PPO) activity, and trypsin inhibitor (TI) activity in this study. The foliage (fourth leaf) was collected during the bioassay for foliar chemical analysis. Five plants were used for each treatment. Leaf samples were ground using liquid nitrogen and then homogenized in a 7% grinding buffer (polyvinylpolypyrrolidone in a potassium phosphate buffer, pH 7). The leaf ground extract (1 mL) was mixed with 100 μL of 10% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in a microtube. The crude extract solution was then centrifuged at 10,000 rpm for 15 min at 4 °C, after which the resulting supernatant was used for determining the enzyme activity. To quantify the amount of protein, a standard curve was prepared using bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA).[4,41,42] Polyphenol oxidase activity was measured based on the procedures of Stout[39] to calculate the formation rate of melanin-like material from catechol. For this assay, 15 μL of a supernatant liquid was mixed with catechol (0.1 M potassium phosphate buffer, pH 8). After mixing for 1 min, an absorbance value below 470 nm was recorded.[8,31,43,44]

For the trypsin inhibitor (TI) assay, we collected and measured the foliage sample (fourth leaf). The leaf sample was ground with liquid nitrogen and homogenized in the extraction buffer (phosphate buffer pH 7.8, 1% PVP, 1% ascorbic acid, 1 mM potassium chloride, 10 mM magnesium chloride, 50 mM EDTA-Na_2) and loaded into the 1.7-mL Eppendorf tube. The crude extract solution was centrifuged at 4 ˚C and at 12000 rpm for 20 min after which the resulting supernatant was used for determining enzyme activity. The TI assay was conducted for three groups; sample, blank, and standard. Each tube of the sample and blank groups were loaded with 50 μL of double distilled water, 75 μL of crude extract, and 250 μL of 2% heated casein solution with 10 mM phosphate buffer (pH 7.6), and incubated at 37 ˚C for 20 min. After incubation, each tube of the sample group was loaded with 250 μL of trypsin solution and 250 μL of double distilled water into the blank group, and incubated at 37 ˚C for 20 min. Each tube of the standard group was loaded with 125 μL of double distilled water, 250 μL of trypsin solution, and 250 μL of 2% heated casein solution with a 10 mM buffer (pH 7.6), and incubated at 37 ˚C for 20 min. Each sample, blank, and standard group was subsequently loaded with 750 μL of 10% TCA and maintained at room temperature for 2 h. Finally, each tube was centrifuged at 4 ˚C, 12000 rpm for 10 min to detect the absorbance value using a spectrophotometer at 280 nm. The TI activity was calculated using the following equation: [(OD280 of standard + OD280 of blank − OD280 of sample)/

OD280 of standard]×100%.[25,42]

5. Insect Growth Bioassay

The insect-feeding performance was conducted to evaluate the effect of PGPMs and plant species on the relative growth rate of *S. litura*. The fourth leaf of each treated plant was removed from the base of the plant by using surgical scissors. The petioles of the leaves were kept in a 2-mL Eppendorf tube with reverse osmosis (RO) water to maintain freshness, after which the leaves were put individually into petri dishes (9 cm in diameter). Third instar larvae of *S. litura* were weighed and individually placed on various treated leaves, and kept in the growth chamber condition (25 ± 2 ˚C, 16:8 h light:dark photoperiod). The larvae were allowed to feed on the foliage for 48 h before being subsequently separated, frozen, oven-dried, and weighed. Fourteen replications (larvae) were included in each treatment. At the same time of the bioassay, fresh weights of 15 third-instar larvae were weighed individually and oven-dried at 45 ˚C. After 1 wk, the dry weights of the larvae were measured again. The average water content of the larvae was used to calculate the initial larval dry weight in the feeding study. The relative growth rate was calculated using the following equation: ((final dry weight of insect-initial dry weight of insect)/initial dry weight of insect)/duration).[11,34,47] The relative growth rate was used as an indicator of insect growth performance.

6. Statistical Analysis

The mean and standard error values were calculated for plant growth (dry weight, leaf area, and water content), relative growth rate (RGR), foliar protein content, polyphenol oxidase (PPO) activity, and trypsin inhibitor (TI) activity. A 2-way analysis of variance (Proc GLM) and Tukey's multiple range test (Version 6.2; SAS Institute Inc., Cary, NC, USA, 1996) were analyzed for comparing the interaction effect between PGPM application and plant species.

III. Results

1. Plant growth performance

Results of this study revealed that the plant growth was affected by plants species and PGPM treatment. The results indicated that foliar dry weight differed significantly among plants species (P=0.0001) that cabbage plants could produce 50% dry weight more than tomato plants did (Fig. 1). Moreover, the PGPM treatment also affected the dry weight markedly (P=0.0361). For the tomato plant, the fungal treatment could produce 50% more dry weight than that of the control treatment. No interaction was detected between plant species and PGPM treatment for the foliar dry weight (P=0.5321). In addition, a significant

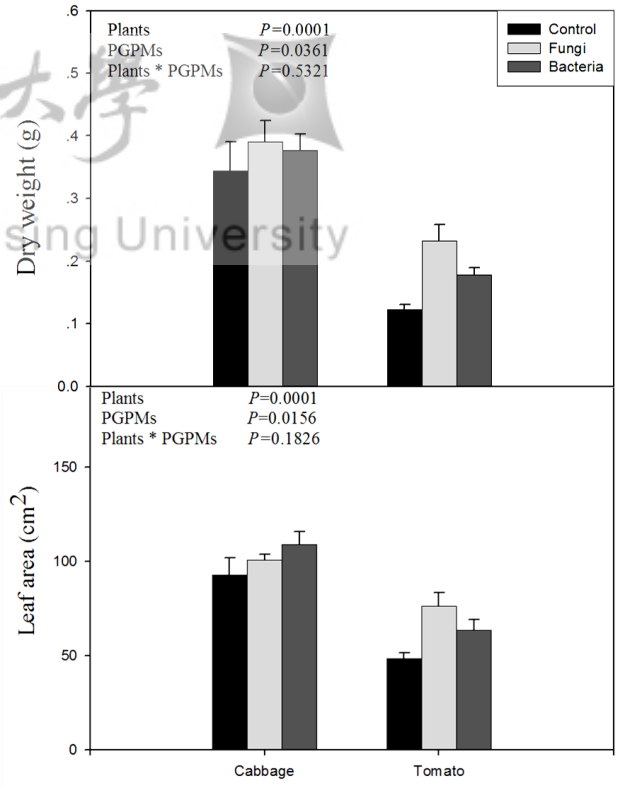

Fig. 1 Plant growth performances of two plant species (cabbage and tomato) treated with or without PGPMs; fungus (*Meyerozyma guilliermondii*), bacteria mixure (*Burkholderia phytofirmans*, *Rhizobium miluonense*, and *Rhizobium lusitanum*). Mean±SE (n=5) (P=0.05, Tukey's test)

difference in leaf area was found for the plant species (P=0.0001) and PGPM treatments (P=0.0156) (Fig. 1). By contrast, the combined effect of plant species and PGPM application on leaf area was insignificant (P=0.1826).

2. Primary nutrient compounds

The plant primary metabolites were influenced by the plant species, PGPMs, and combined plant species and PGPM application. Regarding the water content, the result revealed that the water content was significantly different among plant species (P=0.0001) and PGPM treatments (P=0.0204) (Fig. 2). In addition, the interaction effect between plant species and PGPMs on water content was also significant (P=0.0026). Furthermore, the protein content was significantly affected by plant species (P=0.0001) (Fig. 2). The protein content was also significantly different between PGPM treatments (P=0.0283). A significant interaction effect was also found between plant species and PGPM treatments for the foliar protein content (P=0.0095).

3. Foliar anti-nutrient compounds

The results indicated that the anti-nutrient component differed significantly among the plant species, PGPMs, and interaction between plant species and PGPMs (Fig. 3). The PPO activity was significantly different between plant species (P=0.0004) and PGPMs (P=0.0161). Likewise, the interaction between plant species and PGPMs on PPO activity was also significant (P=0.0278). Tomato plants treated with the mixed bacterial inoculants had a higher PPO activity compared

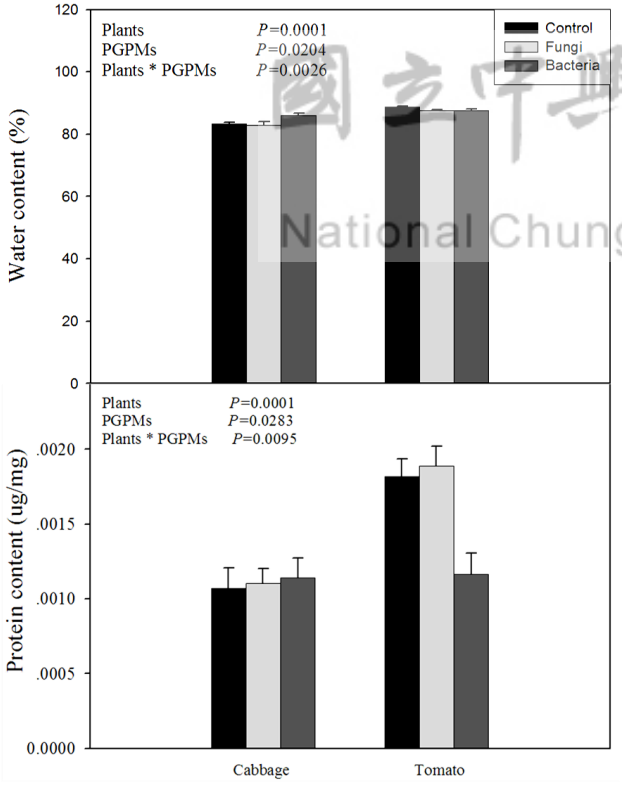

Fig. 2 The protein and water contents of two plant species (cabbage and tomato) treated with or without PGPMs; fungus (*Meyerozyma guilliermondii*), bacteria mixure (*Burkholderia phytofirmans*, *Rhizobium miluonense*, and *Rhizobium lusitanum*). Mean±SE (n=5) (P=0.05, Tukey's test)

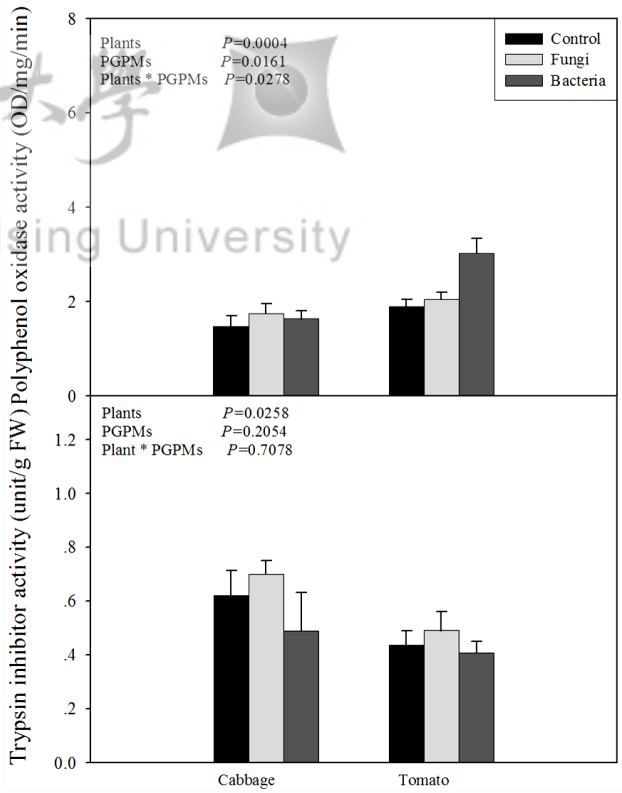

Fig. 3 Anti-nutrient components of two plant species (cabbage and tomato) treated with or without PGPMs; fungus (*Meyerozyma guilliermondii*), bacteria mixure (*Burkholderia phytofirmans*, *Rhizobium miluonense*, and *Rhizobium lusitanum*). Mean±SE (n=4-5) (P=0.05, Tukey's test)

to the other treatments. The TI activity varied significantly between plant species (*P*=0.0258) (Fig. 3). In contrast, the TI activity was not significantly different among PGPM treatments (*P*=0.2054), and the interaction between plant species and PGPMs was not significant (*P*=0.7078).

4. Insect feeding performance

Results of this study revealed that the relative growth rate (RGR) of third-instar larvae of *S. litura* was influenced significantly by the plant species treatment (*P*=0.0001) (Fig. 4). Insects fed on tomato foliage had a lower (50%) relative growth rate than those feeding on cabbage foliage. The relative growth rate of *S. litura*, however, was not significantly different among PGPM treatments (*P*=0.0906) and for the interaction effects between plant species and PGPM applications (*P*=0.8079).

IV. Discussion

This study revealed that both the PGPM treatment and plant species influenced foliar chemistry and insect performance. Moreover, the effects of PGPMs and plant species might interact and affect insect performance significantly through increased foliar polyphenol oxidase activity levels.

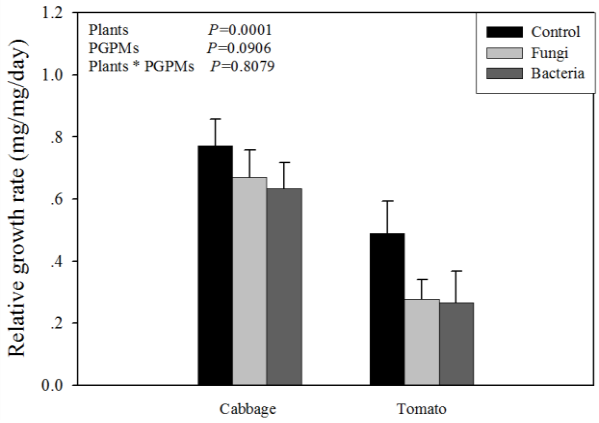

Fig. 4 The relative growth rate of third instar larvae of *Spodoptera litura* on two plant species (cabbage and tomato) treated with or without PGPMs; fungus (*Meyerozyma guilliermondii*), bacteria mixure (*Burkholderia phytofirmans*, *Rhizobium miluonense*, and *Rhizobium lusitanum*). (n=8-14) (P=0.05, Tukey's test)

PGPMs are soilborne microbes that promote plant fitness by directly inducing growth or indirectly by inducing defense.[12,30,32] Moreover, PGPMs influence plant growth, increase nutrient availability, and improve macro - and micronutrients and protein contents of plants.[2,10,14,24] Previous studies have indicated that plants treated with PGPRs induced the activity of PPO, peroxidase, phenylalanine ammonia-lyase, and proteinase inhibitors.[7,12,37] Our study indicated that the application of PGPMs increased the protein content and influenced the PPO activity. Although the PGPMs improve the nutrient uptake of plants, research on the PPO and TI activity remains unverified. Additional studies are required to clarify the effect of PGPMs on the defensive compounds of plants. Previous research has reported that PGPMs induce plant resistance against insect attacks.[27, 30] For example, plants treated with PGPRs affected the feeding of insects such as the cucumber beetle *Diabrotica undecimpunctata howardi* Barber[49], leaf folder *C. medinalis*[32], and leaf miner *Aproaerema modicella*[35]. Our study indicated that the relative growth rate of *S. litura* did not significantly differ in plants treated with PGPMs, suggesting that the effect of PGPMs on insect performance varies depending on microbial species, and biotic and abiotic stresses.

The effect of PGPMs may vary among different plant species and also have different impacts on insect performance.[7,32,33,36] Past studies using different plant species inoculated with PGPMs have revealed that they could increase plant growth and resistance to insect herbivores and plant pathogens.[6,7,26,32,33,35,36,49] Our study indicated that plants treated with PGPMs would produce higher biomass than those without PGPMs. In addition, the effects also varied for the two test plant species.

In summary, both PGPM applications and plant species can affect induced defense responses. The

interaction between PGPMs and plant species might occur and affect certain plant-defensive chemicals; however, this study only provided partial evidence of this interaction. Additional studies are required to clarify the interaction between PGPMs and plant species on plant-induction responses and subsequent insect performances.

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