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評估施用 PGPR (*Bacillus mycoides*)對植物生長及昆蟲表現之影響

Evaluation of PGPR application as bioagent: The effects of *Bacillus mycoides* on plant growth and insect performance

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Abstract

In the conventional agriculture system, high applications of pesticides and fungicides were used widely in order to obtain yields as much as possible. Nowadays, the serious pollutions caused by these synthetic chemicals motivated scientists to find other solutions. Based on previous studies, plant growth-promoting rhizobacteria (PGPR) had potential to be the alternatives. These non-pathogenic microbes not only enhance plant growth but may also indirectly trigger the defensive responses of plants. Through the induced systemic resistance (ISR), pests aboveground may be affected negatively and the better fitness of plants is promised. In this study, the effects of three strains of *Bacillus mycoides*, CHT2401, CHT2402, and CHR001, on plant growth promoting were evaluated. Additionally, the performance of tobacco cutworm (*Spodoptera litura*) and green peach aphid (*Myzus persicae*) fed on treated plants were assessed as well. Cabbage and sweet pepper were first sowed in the soil contained each strain, and the dry weight of plants were recorded 5 weeks later. As for insect performance, both cutworms and aphids were restrained on the specific leaves, after two days, the relative growth rate (RGR), nymph development time and final population size of each treatment were recorded, respectively. In the same time, leaves before feeding and leaves after feeding were collected to analyze enzymes activity such as polyphenol oxidase (PPO) and peroxidase (POD). Results demonstrated that plants under bacterial treatments had significant higher biomass and leaf area than those of the control treatment. However, insects fed on bacteria-treated plants did not show any negative effects. In contrast, the bigger population size was built when cabbage was inoculated with *B. mycoides*. Since there had no significant PPO or POD activity induced, it is assumed that the responses of insect to plant could be influenced by nutrition-related factors. Although these result did not meet the prediction in the beginning, the function of promoting plant growth of these three strains were still valuable. Possibly, with different operating practices, more promising results could be obtained. No matter these beneficial microbes act as biofertilizers or pesticides or both, they should play a significant role in the sustainable agriculture system for sure.

Key words: *Bacillus mycoides*, biofertilizer, defensive chemical compounds, induced systemic resistance (ISR), plant growth-promoting rhizobacteria (PGPR)

中文摘要

在傳統農業中殺蟲劑及殺菌劑被廣泛且高度的使用於防治病蟲害，這些化學合成物質雖顯著提升了農業生產，但同時亦對環境保護造成嚴重的衝擊。相關研究報告指出，在 plant growth-promoting rhizobacteria (PGPR)這些不具致病性的根圈細菌處理下，不僅植株之生長表現獲得促進，此外，亦能夠間接誘導植物之防禦反應。透過 PGPR 誘導出之系統性抗性對地上部的害蟲可能具有防治的效果，進而提升植物之適存度。本實驗之目的為探討 *Bacillus mycoides* 之 CHT2401、CHT2402 和 CHR001 三菌株對植物生長表現之影響，並進一步探討以不同處理之植物餵食斜紋夜蛾及桃蚜所造成之影響。此實驗所使用之植物為甘藍與青椒，分別將其栽植於以 *Bacullus mycoides* 處理過之土壤中，五週後記錄其乾重以比較植株的生長表現。在昆蟲生長發育方面，將斜紋夜蛾及桃蚜限制於植株之葉片上，待其取食一段時間後，記錄斜紋夜蛾之相對生長速率與桃蚜若蚜之發育時間及最終族群之大小。同時將取食前後之處理葉片進行化學防禦物質 polypphenol oxidase (PPO)及 peroxidase (POD)活性分析。試驗結果指出，經 *Bacillus mycoides* 處理過之植株與控制組相較之下顯著擁有較高的生物量和葉面積。然而，取食處理過之植株的斜紋夜蛾並未與控制組有顯著差異；相反的，取食處理過之甘藍的桃蚜，其最終建立出的族群大小顯著較控制組來的大。由於化學防禦物質分析結果沒有顯著差異，故推測兩種昆蟲之表現應是受植株營養差異影響，但尚須針對營養物質作進一步研究。儘管試驗結果並未符合預期，但 *Bacillus mycoides* 對於促進植物生長的效果仍有相當的價值存在，未來可針對操作處理進行調整，以期達到害蟲防治之效果。無論這些有益微生物是扮演生物性肥料亦或是生物性殺蟲劑的角色，其在永續農業上皆有相當的發展潛力存在。

中文關鍵字：Bacillus mycoides、生物肥料、防禦物質誘導系統性抗性 (ISR)、plant growth-promoting rhizobacteria (PGPR)

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Introduction

During the past 50 years, the production of global cereal has doubled, mainly due to a series of technologies from "Green Revolution" (Gomiero *et al*., 2011; Tilman *et al*., 2002). With the improvement of irrigation system, greater input of synthetic fertilizers and pesticides, and new crop varieties development, the incredible high yield was achieved, which reduced food shortage and saved countless people from starvation (Gomiero *et al*., 2011; Tilman *et al*., 2002). However, huge environmental cost also accompanied with the increased food supply (Tilman *et al*., 2001). For example, intensive agricultural activities lead to increasing soil degradation; about 40% of field lands are experiencing overgrazing, soil erosion, or reduced fertility at different levels (Gomiero *et al*., 2011; Montgomery, 2007). In addition, intense applications of fertilizers and pesticides may cause pollution on air and groundwater due to the remainder of those taken up by crops is lost through runoff, leaching, or volatilization (Cassman *et al*., 2002; Tilman *et al*., 2002). Biocides, including pesticide and fungicide especially, not only harm to pests and pathogens, but beneficial organisms may also be influenced negatively in the same time. For example, some beneficial insects like parasitoids and pollinators could be very sensitive to the pollution of ecosystem. Past studies have indicated that abundant synthetic chemicals applied to ecosystem could eventually reduce biodiversity, change species composition, and make crops more susceptible to pathogens and pests (Babalola, 2010; Tilman *et al*., 2002). Furthermore, problems like exposure risks, health hazards, and residue persistence are also need to be considered (Commare *et al*., 2002). Because of these side effects, recent agricultural researches have been focused on finding environment-friendly alternatives for disease and pest management (Commare *et al*., 2002; Zehnder *et al*., 1996).

Utilizing microbes as biofertilizer or bioagent could be a possible solution and potential strategy for sustainable agriculture practices (Figueiredo *et al*., 2011; Jacobsen *et al*., 2004; Kaymak, 2011). A number of soil-borne bacteria were classified into the group of plant growth-promoting rhizobacteria (PGPR) because they were found having functions to help plants overcome biotic and abiotic stresses via growth, vigor enhancement, and induced resistance as well (Commare *et al*., 2002; Herman *et al*.,

2008; Pineda *et al.*, 2010). The term of PGPR has been coined for more than 30 years (Babalola, 2010). In 1978, Kloepper and Schroth first defined PGPR as the microbial communities belowground that benefit plant growth (Kaymak, 2010; Kloepper and Schroth, 1978). Rhizosphere is an active zone of soil surrounding the roots. These non-pathogenic bacteria living in this area will colonize on the surface of roots strongly and directly benefit plants by different mechanisms (Saharan and Nehra, 2011). PGPR may offer plants a better environment with more nutrients and make plants healthier through solubilization of minerals and iron chelation, increasing uptake of the nitrogen, synthesis of phytohormones, producing antimicrobial metabolites or siderophores, or competition with pathogens (Babalola, 2010; Commare *et al*., 2002; Herman *et al*., 2008).

In the very beginning, Suslow *et al*. (1979) reported that when the seeds were treated by specific selected bacterial strains, the yields of sugar beets, potatoes, and radishes increased up to 15, 33, and 144 percent, respectively. Since then, tremendous researches related to bacterial inoculants were conducted (Kaymak, 2011). Until recent years, studies about growth promoting are still in progress. It was found that when *Bacillus subtilis* strain BEB-DN (*Bs*DN), one of PGPR, was treated to tomato plants (*Solanum lycopersicum* cv. Castlemart) with root inoculation (approximately 10^7 colony forming units, cfu, per ml) for overnight, compared to control treatment (water), growth promotion of roots and shoots were evident 3 weeks after inoculation (Valenzuela-Soto *et al*., 2010). Sudhakar *et al*. (2011) also pointed out that the strain *BS3A25* of *B. subtilis* had positive effects on growth of tomato (*S. lycopersicum* cv. PKM1). With treatment of seeds soaking in the bacterial suspension (10^8 cfu/ ml) for 3 hours, germination rate was significantly enhanced to 99%; whereas seeds soaked in the sterile water served as control only had 78% germination. Additionally, no matter what treatments, such as seed treatment, foliar spray, soil drench, or combination of any two methods, were applied, usually treated plants would have significant higher biomass, shoot length, and root length compared to untreated control plants.

To prove the possibility of replacing chemical fertilizers with beneficial microorganisms, Adesemoye *et al*. (2009) conducted an experiment which discussed (1) whether reduced rates of inorganic fertilizer together with PGPR inoculants could produce equivalent yield to plants treated with full rates of fertilizers, and (2) the

minimum application of synthetic fertilizers when microbes were used. In this study, the authors used the mixture of PGPR strains *B. amyloliquefaciens* IN937a, *B. pumilus* T4, and *Bacillus*-based commercial product Plant Growth Activator (PGA) as PGPR treatment and used arbuscular mycorrhiza fungi (AMF) *Glomus intraradices* as AMF treatment. Results showed that the yield of tomato (*S. lycopersicum* cv. Juliet) rose when amount of fertilizer increased; it had the highest production when 100% of the recommended rate was used. However, the same level of yield could be achieved when 70% rates of fertilizer plus PGPR and AMF treatments were applied.

Not only providing enhancement of plant growth, additionally, some PGPR also enable to activate plants' induced systemic resistance (ISR) to protect crops from pathogens and pests injuries indirectly (Akhtar and Siddiqui, 2011; Commare *et al*., 2002; Pineda *et al*., 2010; Valenzuela-Soto *et al*., 2010). Generally speaking, when plants are infested by pathogens or phloem feeders, salicylic acid (SA)-dependent responses will be induced, whereas jasmonic acid (JA)-dependent pathway and ethylene (ET) appears to be activated by chewing insects. Base on different types of herbivores, plants have abilities to adapt themselves by coordinating the production of SA, JA, and ET signaling molecules. The downstream products or the intermediates of these signal pathways allow plants to defense against attacks (de Vos *et al*., 2005; Giordanengo *et al*., 2010; Thomma *et al*., 2001; van Dam, 2009).

Many bacterial species were reviewed as PGPR including *Azotobacter*, *Azoarcus*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Gluconacetobacter*, *Klebsiella*, *Pseudomonas*, *Serratia*, *Rhizobium*, etc. (Kaymak, 2011); whereas ISR is mostly known to be triggered by *Bacillus* spp. particularly (Kloepper *et al*., 2004; Pineda *et al*., 2010). Previous studies showed that ISR may influence pests negatively by changing their behaviors or restricting their development. Zehnder *et al*. (1996) found that when cucumbers (*Cucumus sativus* cv. Straight Eight) were treated with *B. pumilus* strain INR-7 by seeds treatment and root drench (10^8 cfu/ml approximately), spotted cucumber beetles (*Diabrotica undecimpunctata howardi*), the vectors of *Erwinia tracheiphila*, would rather feed on nontreated plants than on PGPR-treated one. The damage of stems and cotyledons and the severity of bacterial wilt were all significantly lower on PGPR-treated cucumbers compared to nontreated ones. On the other hand, root inoculation by brief immersion (10^7 cfu/ml) of tomato with BEB-DN (*Bs*DN) strain of *B.*

subtilis could retard the development of the host, *Bemisia tabaci*. It was reported that although there had no difference in oviposition density, ovipositon preference and nymph number, 28 days after *B. tabaci* infection, the number of emerged pupae obtained from *Bs*DN-treated plants was significantly lower than plants treated by PY9 (a non-PGPR strain of *B. subtilis*), growth medium (GM), and water (control). This phenomenon might be associated with both JA-dependent and JA-independent response because the significant reduction of emerged pupae happened no matter wild type tomato or *spr2* mutant (the mutant with an impaired ability for JA biosynthesis) were tested (Valenzuela-Soto *et al*., 2010). Indeed, these results indicated that *Bacillus* spp. PGPR potentially can play the role as powerful biofertilizer and bioagent in agricultural systems.

Except for their effectives, moreover, some unique characteristics of *Bacillus* spp. strains make them more suitable to be developed as commercial products than other else. Firstly, *Bacillus* spp. survives under natural environment commonly, which can be easily found and isolated from rhizosphere or phyllosphere of different crops. Researchers have isolated strains from cotton (Vijayasamundeeswari *et al*., 2009), potato (Valenzuela-Soto *et al*., 2010), and sugar beet (Bargabus *et al*., 2002) successfully. Secondly, opposite to synthesis chemicals, they are economical and environmental friendly, doing no harm to the ecosystem (Babalola, 2010). The most important feature is that they are more tolerant to unfavorable stresses like desiccation and heat (Figueiredo *et al.* 2011) due to their capability to from endospores under stress. When under stressful conditions, cells will produce endospores to stay dormant and the vigor will be recovered later when the environment is adaptable again, which make them commercially available (Bargabus *et al*., 2002).

In fact, the practical use of *Bacillus*-based biological control agents (BCAs) has advanced in United States, which has great potential in integrated pest management (IPM) system (Jacobsen *et al*., 2004; Kloepper *et al*., 2004). Back in the 1990s, two commercial products Yield Shield and BioYield have been developed and applied in the agriculture broadly for plant growth promoting and disease control (Kloepper *et al*., 2004). Yield Shield particularly received the registration from Environmental Protection Agency (EPA) of America for use on soybean in order to defense against *Rhizactonia solani* and *Fusarium* spp. (Kloepper *et al*., 2004). By 2005, there are 8 more

Bacillus-based commercial products have registered (Figueiredo *et al*., 2011).

Despite of abundant researches demonstrating the positive effects of *Bacillus*-based PGPR on plant disease control, reports focus on pest management are relatively lacking (Jacobsen *et al*., 2004). Take *B. mycoides* as case, especially, previous results pointed out that when sugar beet was treated with *B. mycoides* isolate Bac J (BmJ) by leaf sprays, it reduced the fungal disease Cercospora leaf spot caused by *Cercospora beticola* significantly both in greenhouse and field (Barabus *et al.*, 2002). According to Huang (2008), *B. mycoides* isolate CHT2401 and CHT2402 not only promote different species of plants growth, but prevent cabbage seedlings from damping-off caused by *Pythium aphanidermatum* when the culture medium was inoculated with bacteria in the beginning. Even though *B. mycoides* now is on the process of commercialization, trying to be developed as a product by Advanced Green Biotechnology Incorporation, it only emphasized the positive effects on plant growth and disease control.

Therefore, in this study, the effects of CHT2401, CHT2402, and CHR001 three potential PGPR strains of *B. mycoides* on insects were tested. In addition to the growth promotion of cabbage (*Brassica oleracea*) and sweet pepper (*Capsicum annuum*), the influences of treated plants on herbivores were also evaluated. Moreover, two types of feeders were used in this study, chewing insect tobacco cutworm (*Spodoptera litura*) and sucking insect green peach aphid (*Myzus persicae*). Last but not the least, in order to figure out if there had any defensive response (JA-dependent or SA-dependent pathway) was induced when bacteria-treated plants were attacked by insects. Here the induced defense related proteins, such as polyphenol oxidase (PPO) and peroxidase (POD) were analyzed. In short, the objective of this research is to evaluate the possibility of using *B. mycoides* on the insect pest management practice.

Materials and Methods

1. Plant culture

The seeds of both cabbage (*Brassica oleracea* cv. Summer Summit) and sweet pepper (*Capsicum annuum* cv. Green Star) were purchased from local seed company (Known-You Seed Co., Ltd. Kaohsiung, Taiwan). After treating with 45℃ warm water for 30 minutes to do surface sterilization (Tan *et al*., 2011; Yadav *et al*., 2010), seeds were sown into 3-inch plastic pots contained Bas Van Buren No.3 (BVB No.3, Masland, Netherlands) culture medium soil. Plants were maintained in greenhouse under the condition of temperature 25±2℃, photoperiod 16 h light/ 8 h dark during whole experimental period and were watered every day.

2. Insect rearing

Green peach aphids (*Myzus persicae*) and tobacco cutworms (*Spodoptera litura*) were used in this study to assess the effects of plants' treatments on insects' performance.

2.1. Green peach aphid

Green peach aphids were collected from cruciferous crops planted in the field located in Taichung, Taiwan. Aphid population was kept in the plastic cage (Bug Dorm-1, 30 × 30 × 30 cm^3, mesh size 100 × 100, Mega View Science Co., Ltd. Taichung, Taiwan) in the incubator (24℃, 16 h light/ 8 h dark photoperiod, 70% r. h.; Yuh-Chuen-Chiou Industry Ltd. Kaohsiung, Taiwan) and fed with radish plant (*Raphanus sativus* cv. Fu-Ho).

2.2. Tobacco cutworm

The source of tobacco cutworms was from the population that kept in the Insect-Plant Interaction Laboratory, Department of Entomology, National Chung Hsing University (NCHU), Taichung, Taiwan. Cutworms grew in the incubators under the controlled environment (25℃, 16 h light/ 8 h dark photoperiod, 70% r. h.) all the time. Since they hatched out from eggs, the artificial diet was offered to them to feed on until experiments. The ingredients of the artificial diet were mainly 550 ml reverse osmosis water (RO water) mixed flowering bean meal (150 g), wheat germ powder (55 g), and yeast powder (60 g). In order to make the diet solid but flexible, boiled agar (37.5 g) in 600 ml RO water was fully stirred into the liquid introduced above. Until the temperature went down, the nutrients of L-cysteine (0.6 g, Sigma, USA) and L-ascorbic acid (6 g, Santiku, Japan) in RO water (50 ml), and the preservatives of sobic acid (1.5 g, Sigma, USA) and Methyl-p-hydroxbenzoate (1.75 g, SIGMA, USA) in the solution of alcohol (30 ml) were added and mixed completely. In this study, cutworms were used when they developed to 4^{th} instar.

3. Bacterial strain and preparation of bacterial treatment

3.1. The source of bacterial strains

Three *Bacillus mycoides* strains, CHT2401, CHT2402, and CHR001 were isolated from the soil collected from central area of Taiwan and preserved by Laboratory of Plant Disease Management, Department of Plant Pathology, NCHU, Taichung, Taiwan (Huang, 2008). Each strain later was cultivated on the nutrient agar (NA, Difco™ Becton, Dickinson and Company, MD, USA).

3.2. Preparation of bacterial suspension and broth

In this study, two bacterial formulations were used to see the different effects caused by *B. mycoides*. The bacterial suspension only (named as ST-treatment in this study) and bacterial broth (named as SM-treatment in this study) were used. For the preparation of ST group (ST-CK, ST-CHT2401, ST-CHT2402, and ST-CHR001 included), each bacterial strain was first washed with sterile water directly; then the liquid contained *B. mycoides* was diluted and adjusted to the concentration of 1×10^8 cfu/ml ($OD_{620} = 1.0$) approximately through spectrophotometer (GENESYS 2 and 5 spectrophotometer, Spectronic Unicam, USA). The accurate number of cells was unable to measure owing to the chain-forming of this microorganism (Bargabuts *et al*., 2002). Sterile water was taken as control (ST-CK).

As for SM group (SM-CK, SM-PCK, SM-CHT2401, SM-CHT2402, and SM-CHR001 included), considered the spontaneous proliferation of bacteria, the soybean milk was used as the culture medium for the fermentation purpose. Soybean meal purchased from food material firm (Yong-Cheng Food Co., Ltd. Taichung, Taiwan) was first dissolved in RO water with the ratio of 10% w/v. After heating for 30 minutes, the soybean milk was filtered through gauzes (4 layers, mesh size 26 × 26 each layer). Flasks contained filtered soybean milk were placed into autoclave (121℃, 1.5kg/cm^2, for 20 minutes; EA-625T, Trident Medical) to sterile before inoculation. For inoculating *B. mycoides* strains to steriled soybean milk, the suspensions of CHT2401, CHT2402, and CHR001 ($OD_{620} = 0.3$) need to be prepared in advance. When sterile soybean milk were cooled down to the room temperature, the suspension of each strain with adjusted concentration, and sterile water (used as positive control, PCK) were allowed to be loaded with the ratio of 10% (v/v). The process of inoculation should be operated inside the laminar flow to avoid any possible pollution. Finally, flasks were set on an automatic shaker [100 rpm; Orbital shaker incubator (Hotech); Model: 706R] for 3 days at 30℃. Sterile water was taken as control (SM-CK). This protocol was modified for Huang (2008).

4. Effects of *Bacillus mycoides* on plant growth

In order to evaluate the effect of different strains of *B. mycoides* and ways of

applications of these bacteria on plants' growth, two bioassays were conducted. First application method is using seed coating. Seeds were soaked in 9 different treatments (Table 1) for 30 minutes and planted into the 3-inch plastic pots. The second method is applying through soil mixture. The suspension of ST group and the broth of SM group were added into the soil BVB No. 3 with the ratio of 5% (v/v), respectively, in 9 plastic pots. Water was later added into pots as well to control the water content (15~20%). After fully stirring, soil mixed strains and water together was stuffed into 3-inch plastic pots and seeds of cabbage and sweet pepper were sown into it. Plants grown under the controlled environment as described above were collected 5 weeks after sowing for recording growth index, including dry weight, leaf area, and water content. Five replications per treatment were applied.

5. Effects of *Bacillus mycoides* treated plants on insect performance and preference test

5.1. Effects on tobacco cutworm

To understand the effects of different bacterial strains on tobacco cutworms, two experiments were designed: the preference of cutworms to the leaves of treated cabbage or sweet pepper *in vitro*, and the growth performance when they fed on the specific leaf of treated plants *in vivo*. Plants for insect-related experiments were all treated with soybean milk contained *B. mycoides* (SM treatments) though soil mixture. For the former experiment, two sections had been done. The first one was that leaves removed from plants were collected and set on the plate directly (30 × 30 cm^2, Mega View Science Co., Ltd. Taichung, Taiwan); one plate had 4 treated leaves (PCK, CHT2401, CHT2402, and CHR001) placed at 4 corners randomly. Ten 4^{th} instar larvae in petri dish (diameter is 4 cm) were set at the center and whole plate was cover by food warp to avoid escape (Figure 1). The number of cutworm on each leaf was recorded every 30 minutes within 2 hours, 6 replications. Another section was similar to the first one, but before set on the plate, leaves were sprayed by bacterial suspension (1 × 10^8 cfu/ml

approximately) additionally. Since there had wax on the surface of cabbage, the sprayer (S-240, Germany) was added in the solution.

As for the experiment of growth performance, since it had the difference of growth rate between cabbage and sweet pepper, the larvae just exuviated and entered to 4th instar were used to feed on 2nd newest and fully spread leaf of 4-week-old cabbage and 4th newest leaf of 5-week-old sweet pepper under SM treatments belowground. After 48 hours, relative growth rate (RGR) and feeding area was calculated later by leaf planimeter (Figure 2). ten replications per treatment were conducted.

$$RGR = \frac{\text{Added weight within development time (dry weight)} \;/\; \text{Original weight (dry weight)}}{\text{Development time (day)}}$$

5.2. Effects on green peach aphid

Besides assessing the effects on chewing insect like tobacco cutworm, the influences of *B. mycoides* on aphid, sucking insect, were tested as well. To assess the effects of treated plants on aphids feeding, aphid growth performance bioassay was conducted and evaluated. In this study, five SM treatments mentioned previously were tested. Four-day-old wingless nymphs were removed from radish tenderly to the newest and fully spread leaf of 4-week-old cabbage and the 3rd newest leaf of 5-week-old sweet pepper, one nymph per plant. The aphid on the leaf was given a limited space by covering a net in order to avoid it from escape. The existence of aphid was checked every day. In addition, the date the nymph became an adult and the occurrence of next generation were recorded. Three days after first descendants coming out, whole aphid population was counted, ten replications were conducted for each treatment.

In addition to the performance of individual aphid under different treatments, it was also interested to know the preference of them when they were exposed to the environment with different treated plants. The preference test of aphids was designed similarly as the one of cutworms; but 30 aphids were used and the number was recorded after 3, 6, 12, and 24 hours, 6 replications were conducted.

6. Chemical analysis

In order to find out which factor that exactly affected the performance of cutworms and aphids, protein content was considered as a nutrient index and two protein based defensive compounds, polyphenol oxidase (PPO) and peroxidase (POD) were considered as defense index. Plants for chemical analysis were all treated with soybean milk contained *B. mycoides* (SM treatments) though soil mixture. The positions of tested leaves collected from cabbage were not always the same owing to different design of each part of experiment (Table 2).

6.1. Analysis of protein content

Leaves collected from plants were weighed first, and then with liquid nitrogen, leaves were grinded by a mortar and pestle. Based on fresh weight of the leaf, 7% grinding buffer [polyvinylpolypyrrolidine in potassium phosphate buffer (K-P buffer), pH=7] was added into mortar (with the ratio of leaf: buffer = 150 mg~250 mg: 1 ml) when leaf completely became powdery. One ml leaf-buffer-mixed liquid and 100 µl surface active agent Triton X-100 (Sigma, USA) were loaded into a micro tube. The crude extract solution was centrifuged at 4℃, 12,000 rpm for 20 minutes (Refrigerated centrifuge, Hettich, Zentifugen EBA 12R). The supernatant was collected for quantification of protein and analysis of peroxidase and polyphenol oxidase subsequently.

To quantify the amount of protein, a standard curve was needed, which was made through bovine serum albumin (BSA, Sigma, USA) as the standard. A series of different concentrations of BSA in K-P buffer solution was prepared (0, 8, 16, 24, 32, 40 µg / µl) for the conversion of protein amount of sample. After loading 10 µl supernatant liquid of each sample and 50 µl of each 6 standards, five µl, 0.1 N HCl, 40 µl double distilled water, and 1750 µl, 20% protein assay dye reagent (Bio-Rad 500-0006), were all added into the eppendorf. Five minutes after coloring, 200 µl liquid of each tube was loaded into a 96 micro well plate (NUNC, 442404) and detected the absorbance value under 570nm by ELISA reader (Thermo, Multiskan EX). Compared with the BSA standard

curve, the protein amount of leaf could be calculated easily (Bradford, 1976). The content of protein in each sample was calculated by following equation:

$$\text{Protein content (μl/mg)} = \frac{\text{Concentration of protein (μg/μl)} \times 10^3}{\dfrac{\text{Leaf dry weight (mg)}}{\text{Added liquid for grinding (ml)} + \text{water content of leaf (ml)}}}$$

6.2. Activity of polyphenol oxidase (PPO)

After protein analysis, 10~100 μl supernatant liquid was mixed with 10 mM K-P buffer (pH=8) contented catechol (Sigma, USA) and then read the absorbance value under spectrophotometer (OD_{470}). The value after conservation was expressed by the unit of △OD min^{-1} mg^{-1} (Ryan *et al*., 1982).

6.3. Activity of peroxidase (POD)

The process is as same as the analysis of PPO, but the supernatant liquid was mixed with K-P buffer (pH =8) contained 0.02 mM H_2O_2 and 5 mM guaiacol (Sigma, USA). After detecting the absorbance value under $OD_{470,}$ the activity was shown by the unit of △OD min^{-1} mg^{-1} (Ryan *et al*., 1982).

7. Statistical analysis

All data were analyzed through software of SAS System V.8 (SAS Institute Inc. Cary, NC, USA). One-Way ANOVA and Fisher's LSD test ($p < 0.05$) were used to compare

the results of biomass, leaf area, RGR, duration time of aphid, final aphid population, and chemical analysis among different treatments. Furthermore, when compared enzymes activity of cabbage without insect feeding to damaged plants, T-test was used to check the difference between two treatments. As for the preference test of insects, Percentage data were arcsine-square-root transformed in advance to analyze.

Results

1. Effects of *Bacillus mycoides* on plant growth

1.1. Plants treated bacterial strains by seed coating

With soaking in the suspension (ST treatments) of *Bacillus mycoides*, the results showed that the dry weight of cabbage had no significant difference between any two treatments (Figure 3a). Also, although the CHR001 treatment made the highest dry weight of sweet pepper (0.322 g), CHT2401 (0.250 g) and CHT2402 (0.264 g) treatments did not show significant difference compared to control (CK, 0.236 g) treatment (Figure 3b). On the other hand, when seeds of cabbage and sweet pepper were treated by broth coating (SM treatments), the highest dry weight was achieved with the extra application of bacterial strains. However, control and positive control (PCK) treatments still offered the equivalent effects on growth promotion (Figure 4).

1.2. Plants treated bacterial strains by soil mixture

With the ST application, the dry weight of cabbage plants does not have significant difference among control (0.534 g), CHT2401 (0.522 g), and CHT2402 (0.474 g) treatments. This phenomenon was observed in sweet pepper as well; dry weight of each treatment was equivalent to each other without significant difference (Figure 5). Nevertheless, when the soil was mixed with bacterial broth (SM treatments) ahead, plant growth showed significant differences evidently (Figure 6). Cabbage under CHT2402 treatment got the marked highest dry weight (0.750 g), and then CHR001 (0.622 g), CHT2401 (0.518 g), PCK (0.432 g), and CK (0.344 g). Compared to the control, the dry weight was increased from 0.5 to 2 times by other 4 treatments ($p < 0.0001$; Figure 7a). The significant difference among 5 SM treatments also happened to sweet pepper. The dry weight was enhanced the most with CHT2401 and CHR001 treatments (0.372 g and

0.356 g); plants under control treatment got the lowest performance (0.128 g; Figure 7b). No matter cabbage or sweet pepper, the results of leaf area had the same trends as the dry weight ($p < 0.0001$; Figure 8).

2. Effects of *Bacillus mycoides* treated plants on insect performance and preference

2.1. Effects on tobacco cutworm performance

After 4th instar larva fed on treated plants for 2 days, even though the highest RGR (0.87) happened to cutworms fed on CHT2402-treated cabbage, there had no significant difference among the 5 treatments (Figure 9a). Additionally, the performance on sweet pepper seemed not different either (Figure 9b). However it was noteworthy that when cutworms fed on cabbage with control treatment, the biggest feeding area was consumed (Figure 10).

2.2. Effects on tobacco cutworm preference

The results showed that under a covered environment, no matter treated leaves had additional spray or not, cutworms did not have specific preference among 4 different treatments of cabbage and sweet pepper since the responded number has no significant difference (Figure 11, Figure 12).

2.3. Effects on aphid performance

When 4-day-old nymphs were restricted on cabbage with different treatments, the time they spent becoming adults didn't vary significantly (Figure 13). The development time of each treatment was between 3.3 and 4.2 days. However, after 5 days of

reproduction, the final population size had dramatically different ($p < 0.0001$; Figure 14). Population under control and positive control treatments only had 3.14 and 10.00, respectively. In contrast, aphid number on CHT2401, CHT2402, and CHR001 treated leaves were 3 times to 4 times more than that of the control.

2.4. Effects on aphid preference

According to the analysis, among 4 different treatments of cabbage or sweet pepper, the preference choice of aphids did not have any significant difference even though the lower average happened to CHT2402 treatment (Figure 15). Although the aphids seemed attractive to PCK treatment (sprayed with water) when sweet pepper leaves were sprayed with bacterial suspension, there still had no significant difference between any two treatments (Figure 16).

3. Chemical analysis

3.1. Before insect feeding

When cabbage was 4-week-old, the leaves were collected freshly and the defensive compounds such as polyphenol oxidase (PPO) and peroxidase (POD) were analyzed. Results showed that plants had the highest PPO activity under PCK and CHT2401 treatments (16.756 and 15.75); other three treatments had no difference (Figure 17). As for POD, the lowest activity still happened to CHT2402 and CHR001 treatments, but cabbage under CK treatment achieved the higher level (Figure 18).

3.2. After insect feeding

After cutworm feeding, the PPO activity of cabbage leaves had no any significant difference in contrast to 4-week-old, undamaged plants (Figure 19). However, when the

enzyme activity was analyzed by the method of One-Way ANOVA and Fisher's LSD test ($P \leq 0.05$), cabbage under control treatment had the highest PPO activity (Figure 20). Reversely, POD activity was enhanced significantly after aphid feeding except CHT2401 treatment (Figure 21). However, when the enzyme activity was analyzed by the method of One-Way ANOVA and Fisher's LSD test ($P \leq 0.05$), there had no significant difference among CK, PCK, CHT2402, and CHR001 treatments; all of 4 were higher than CHT2401 treatment (Figure 22).

Discussions

Based on the results, it could be found that when seeds of cabbage and sweet pepper were treated with suspension (1x 10^8 cfu/ml) of *Bacillus mycoides* strains CHT2401, CHT2402, and CHR001, dry weights of plants were higher than that of the control treatment, yet the difference was not significant (Figure 3). The similar results also happened to seeds soaked in SM treatments (Figure 4). Combined two experiments, it was assumed that these three strains all had the potential for promoting plants growth; however, way of operating could be very critical. Sudhakar *et al*. (2011) soaked tomato seeds into suspension of *B. subtilis* (1x 10^8 cfu/ml) and the dry weight were enhanced significantly compared to control treatment. Notably, they had treated seeds for 3 hours with gentle agitation rather than a short period of time. Interestingly, although the soybean milk here was taken as nutrient, since soybean was the source of nitrogen, the average dry weight of each treatment of SM was lower than ST treatments (Figure 3; Figure 4). Chu *et al*. (1984) pointed out that when fertilizer with a normal rate was placed in touch with potato, the emergence was delayed. In this case, the possible reason was that soybean milk with high concentration (10%) contacting with seeds directly might cause fertilizer injury, too.

On the other hand, when the bioassay protocol of Huang (2008) was followed, growth of plants treated with bacteria containing soybean milk was increased significantly (Figure 7). Strains of CHT2401, CHT2402, and CHR001 were possessed of being PGPR. Yet, the similar trend did not show up within bacterial suspension treatments (Figure 5). Previous study indicated that applied organic fertilizer to soil could affect the bacterial population positively (Chang *et al*., 2007). From these results, it was speculated that as an organic material, the presence of soybean milk played an important role. One of the possible mechanisms behind this phenomenon was that strains of *B. mycoides* might build a sturdy colony belowground and then promote the growth by increasing nitrogen uptake of plants. The higher dry weight also guaranteed bigger leaf area, which may promise stronger photosynthesis and higher yield in the future (Figure 8).

In the beginning, it was predicted that the preference of insects might be affected by

different treatment and the special smell of these three strains. That's the reason why leaves were treated suspension spray additionally. However, from results of insect preference, both cutworms and aphids had no difference between any two treatments (Figure 11, 12, 15, and 16). During the process of preference test, it was found that once they chose the specific leaf, they seldom gave it up and then search for another one. Instead, if one of them made the decision in advance, others seemed to be attracted soon after. It is believed that although the volatiles which induced by herbivores were regarded as the indirect defenses, they could also be the attractants to the herbivorous arthropods (Dicke and Baldwin, 2010). To reveal the functions and effects of the spray treatments on insects, field studies are needy further.

Though organic materials such as microorganisms and compost were thought to increase the resistance of plants, the research of Little *et al*. (2011) showed that green peach aphid had no significant preference to leaves treated with different concentration of vermicompost after 24 hours. Nevertheless, the nymphs deposited by an adult were significantly more on the control treatment than vermicompost treatment. Compared their work to this study, green peach aphids did not have specific choice either, but the results of population size was just opposite to previous study. Here the fecundity of adult aphid fed on bacterial treated leaves was significant higher than those of control or positive control treatments (Figure 14). Even though POD activity was induced significantly by aphid feeding (Figure 21), which was consistent with the work done by Sudhakar *et al*. (2011), significant difference did not be reflected among the 5 treatments (Figure 22), so there should have other factors involved. In the study of Little *et al*. (2011), nutrient solution was applied to all plants one day a week to make sure that nutrition deficiency would not influence the insect responses to non-vermicompost ones. Combinations of the above, nutrition could be considered as a factor which led to different results in this experiment. To understand the influence of it, more relative analyses could be done in the future, such as nitrogen content, total non-structural carbohydrates, and C/N ratio may be included.

As for the performance of cutworm, because the significant difference of polyphenol oxidase activity (PPO activity) could not be detected clearly among 5 treatments (Figure 19), one might suspect that cutworms fed on bacterial-treated leaves had a better growth. To one's surprise, when they were forced to feed on specific plants,

the RGR eventually showed no difference (Figure 9a), which meant that they performed equally no matter *B. mycoides*-treated or non-treated cabbage they fed on. One possible theory "compensatory feeding behavior" could be used to explain this phenomenon. When insects infest plants that are not so nutritious, one of their strategies is increasing the food uptake in order to overcome this difficulty, so that they can maintain the fitness for future development (Schoonhoven *et al*., 1998). For instance, after feeding on milkweed plants (*Asclepias* spp.) that applied lower nitrogen fertilizer, larva of monarch butterfly (*Danaus plexippus*) were larger than those fed on higher-nitrogen-fertilizer treated plants (Lavoie and Oberhauser, 2004). In this study, it was also found that the biggest feeding area happened to cabbage under control treatment, which might support hypothesis further (Figure 10). On the other hand, Figure 20 showed that plants under control treatment had the highest PPO activity compare with positive control treatment, which just had a negative relationship between plant growth promotions (Figure 7a). This result more or less supported the concept of "growth-differentiation balance hypothesis" (Lorio, 1986). When resources are limited (without nitrogen, for example), photosynthesis of cabbage will be confined simultaneously. To adapt the deficiency, plants may allow themselves to synthesize elements to defensive secondary compounds to against stresses. However, the performance of cutworm was not affected negatively (Figure 9a); perhaps 4th instar larva were not as sensitive as larva at 1st or 2nd instar stage.

Results of this study revealed that apart from the plant growth promoting, *B. mycoides* isolates CHT2401, CHT2402, and CHR001 belowground treatments may also benefit pest feeding aboveground. As the liking element, plant plays an important role in microorganism-plant-insect interactions. This relationship among multi-trophic levels is controversial. On the one hand, defensive compounds of plants could be induced through PGPR treatments against herbivores; on the other hand, however, enhanced plant growth represents increase of food supply and improvement of nutrition quality in the same time (Pineda *et al*., 2010). One example is the study from Kempel *et al*. (2009). They indicated that when white clover *Trifolium repens* was treated by rhizobia *Rhizobium leguminosarum* through the air and soil, plants got higher biomass; cutworm (*S. littoralis*) and aphid (*M. persicae*) had better performance as well. For this reason, it is necessary to detect the concentrations of nitrogen and other nutritious components

occurred in the leaf for the future studies.

Even so, it is not suitable to overthrow the possibilities of ISR triggered by these three strains, because in this study, still many operating practices could be modified and revised. For instance, method and frequency of treating need some work. Except for soil mixture, plants can also be treated by the way of seed coating, root application, foliar spray, soil drench, and even combinations of any two or three of the above methods (Sudhakar *et al*., 2011; Valenzuela-Soto *et al*., 2010). In addition, treated plants more frequently is also an effective way. Bargabus *et al*. (2002) proved that when sugar beet was treated with *B. mycoides* isolate Bac J (BmJ) once every 14 days by foliar spray, similar disease (Cercospora leaf spot) control was achieved to fungicides. Additionally, increase activity of chitinase, β-1, 3-glucanase, and peroxidase (POD) were detected within plants treated with Bm J. Obviously, a lot of the rests can be discussed resulted from these cases.

Conclusions and future prospects

Combinations of all above, this study showed that *B. mycoides* strains CHT2401, CHT2402, and CHR001 acted as PGPR since they had positive effects on plant growth. However, with the better growth performance, plants became more attractive to herbivores simultaneously. Although it seemed difficult to regard these microbes as the alternatives of pesticides, they were qualified to be developed as biofertilizers without a doubt. These beneficial microorganisms play an important role in the integrated pest management, which also take a place in sustainable agriculture system. Moreover, it is believed that there will be more use of them in the crop production and disease and pest control. For the future prospect, besides further researches and developments, building a fully-formed system about PGPR is more important and needful. By this systemic structure, farmers are able to follow the illustrations, knowing what to use and how to use, which will be the most valuable contributions of relative studies.

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Table 1. Illustration of 9 different treatments used in this study

Group	ST				SM				
Treatment	CK	CHT2401	CHT2402	CHR001	CK	PCK	CHT2401	CHT2402	CHR001
Description	Sterile water	Sterile water + CHT2401	Sterile water + CHT2402	Sterile water + CHR001	Sterile water	soybean milk + Sterile water	Soybean milk + CHT2401	Soybean milk + CHT2402	Soybean milk + CHR001

Table 2. Illustration of the leaves sources of the chemical analysis

	Plant	Plant age	Location of sample
Before feeding	Cabbage	4-week-old	1st and 2nd newest leaves
After cutworm feeding	Cabbage	4-week-old	The leaf behind the fed one
After aphid feeding	Cabbage	4-week-old	The leaf aphid feeding on

Figure 1. The design of the cutworm preference test.

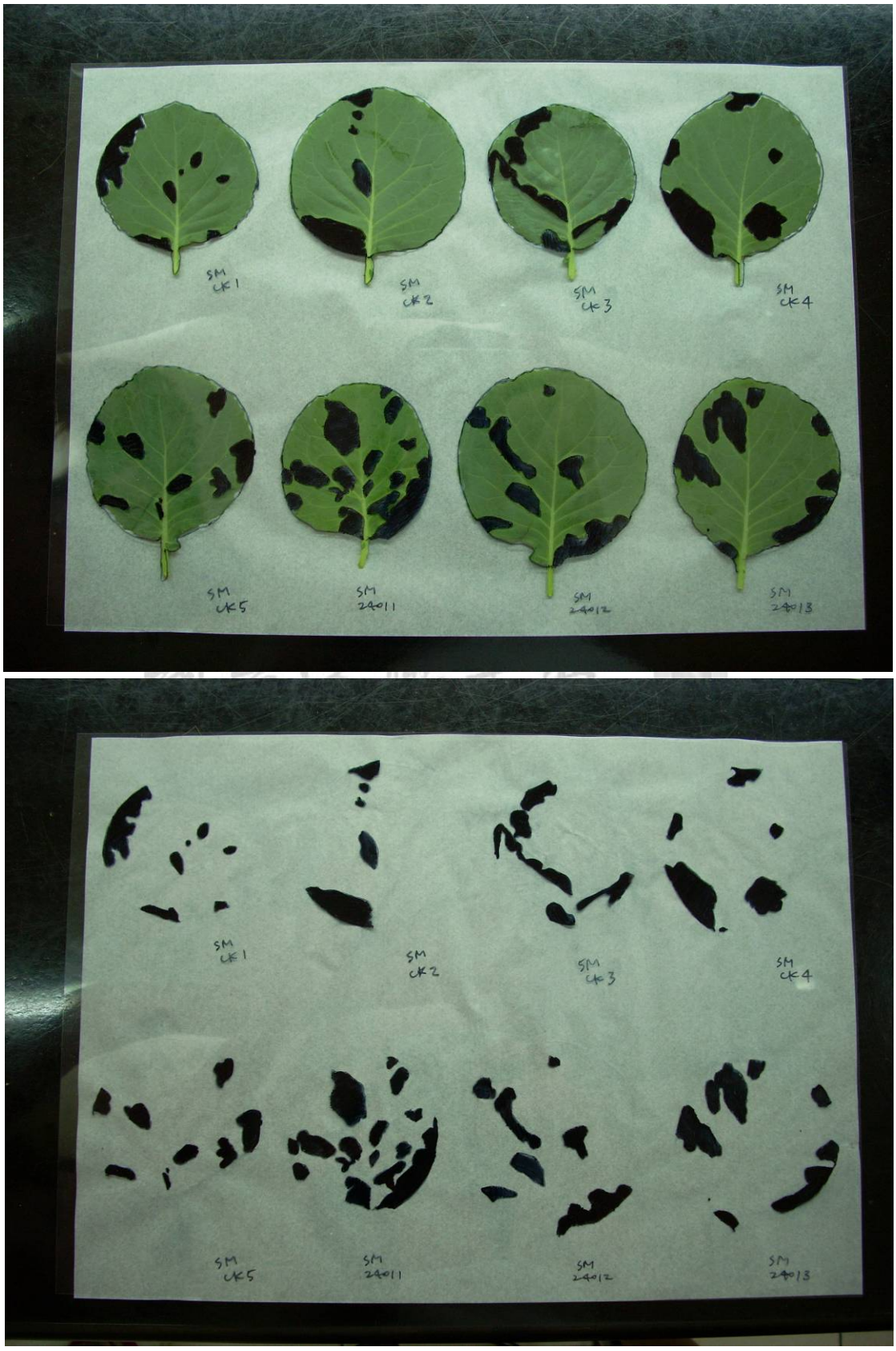

Figure 2. The cabbage feeding area of 4th instar tobacco cutworms.

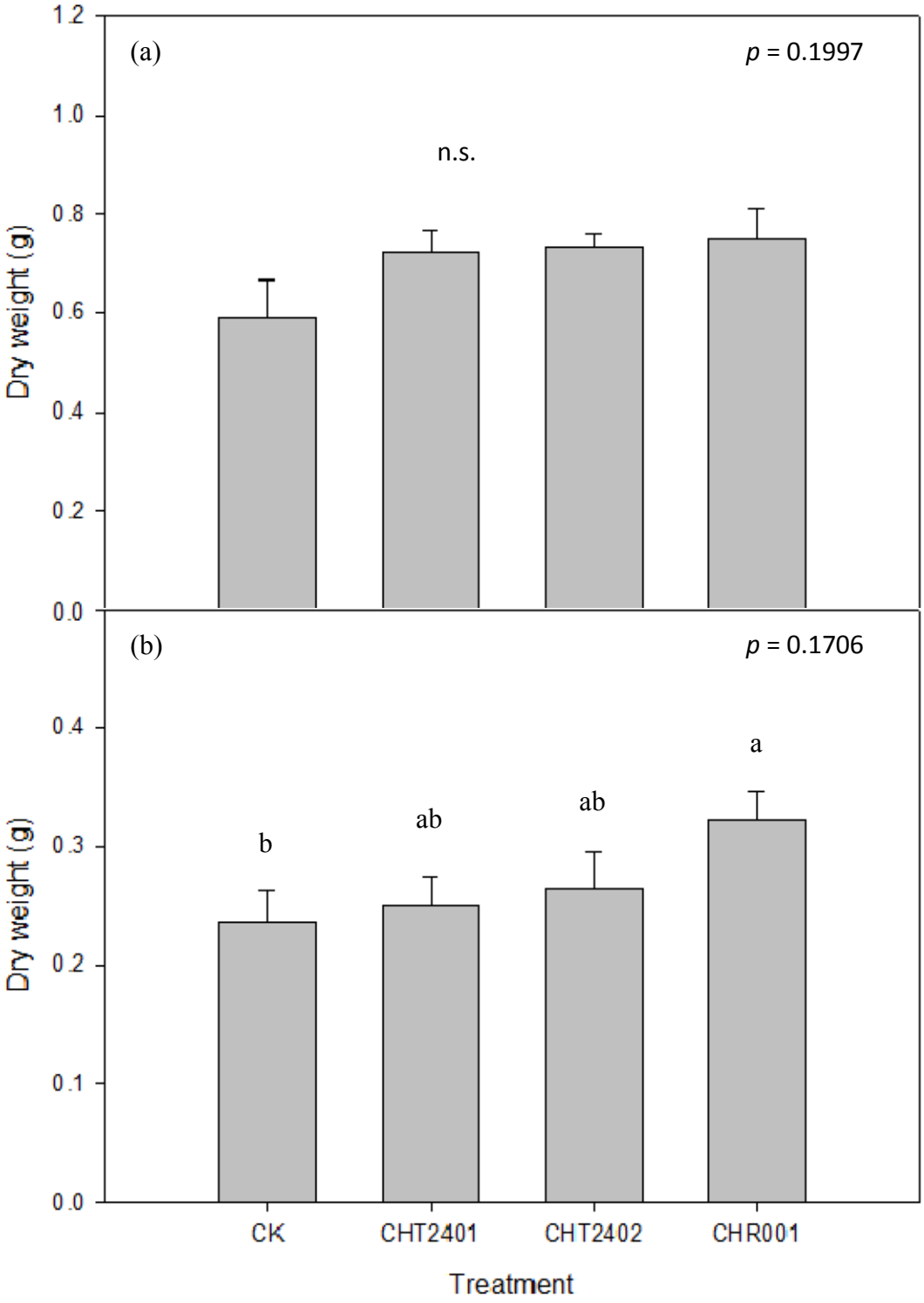

Figure 3. The dry weight of plants under different ST treatments by seed coating. (a) cabbage. (b) sweet pepper. Bars without the same letters are significantly different ($p < 0.05$). CK: water; CHT2401, CHT2402, and CHR001 mean different strains of *Bacillus mycoides* suspension.

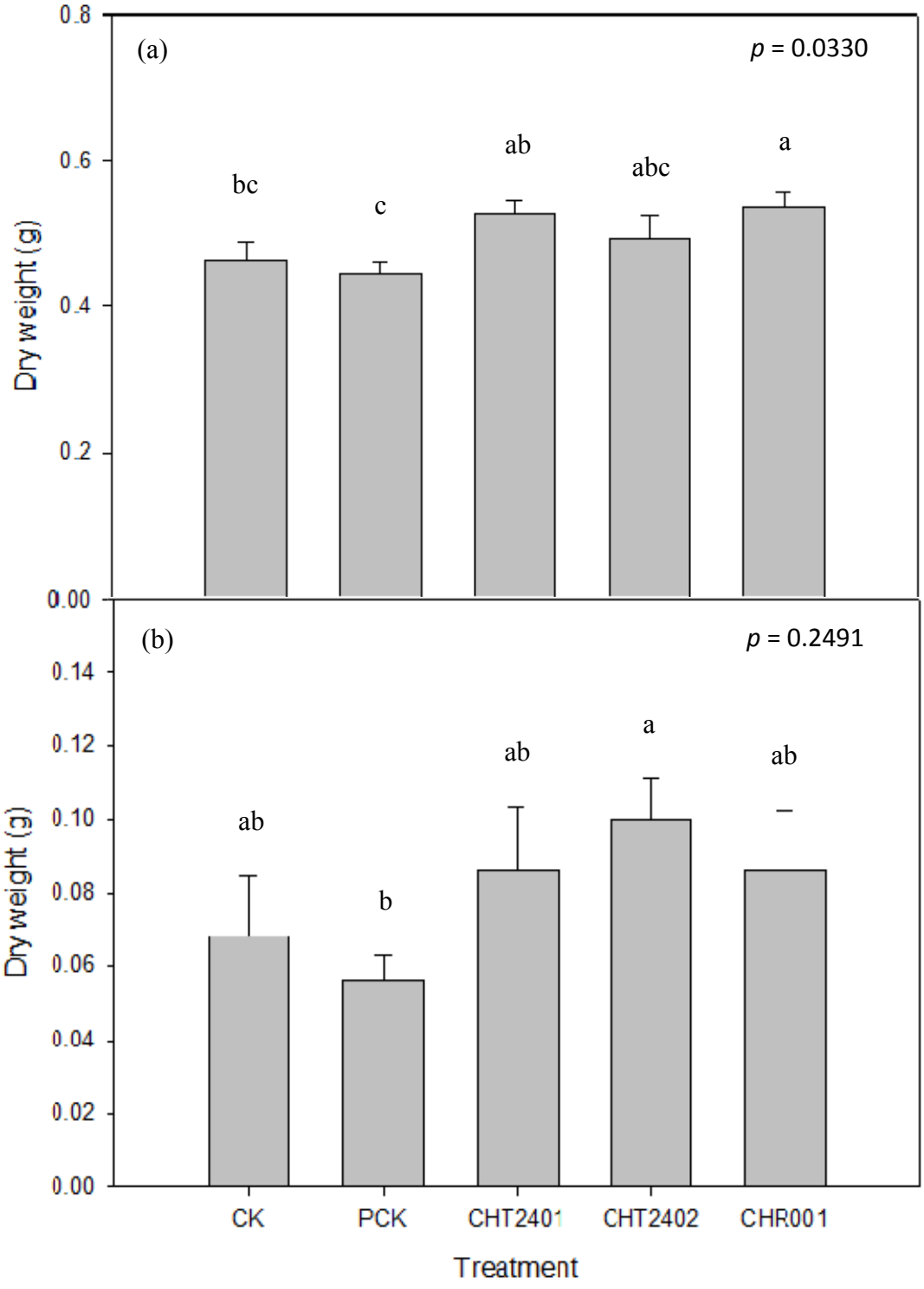

Figure 4. The dry weight of plants under different SM treatments by seed coating. (a) cabbage. (b) sweet pepper. Bars without the same letters are significantly different ($p < 0.05$, Fisher's test). CK: water; PCK: soybean milk; CHT2401, CHT2402, and CHR001 mean different strains of *Bacillus mycoides* cultured in soybean milk.

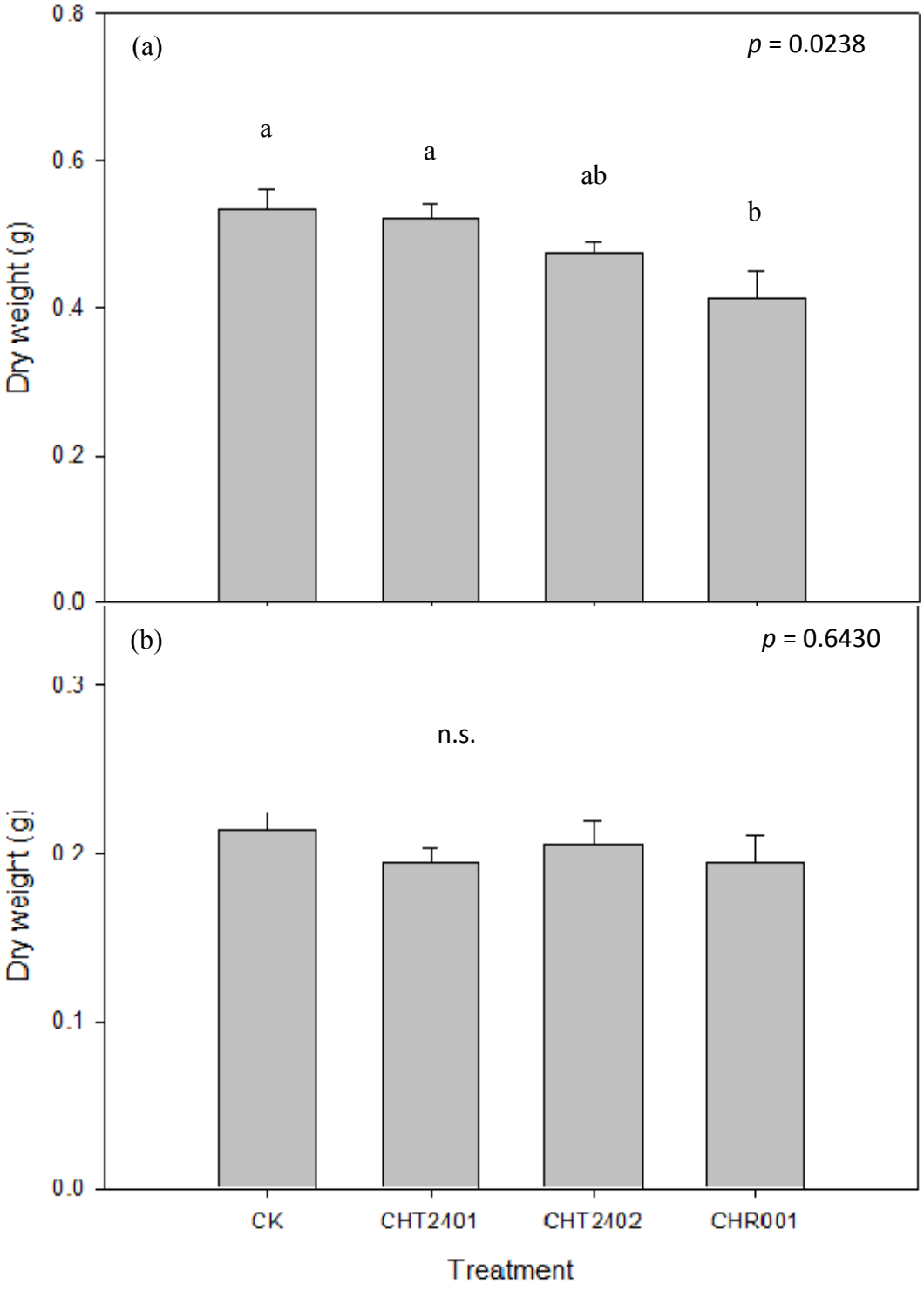

Figure 5. The dry weight of plants under different ST treatments by soil mixture. (a) cabbage. (b) sweet pepper. Bars without the same letters are significantly different ($p < 0.05$). CK: water; CHT2401, CHT2402, and CHR001 mean different strains of *Bacillus mycoides* suspension.

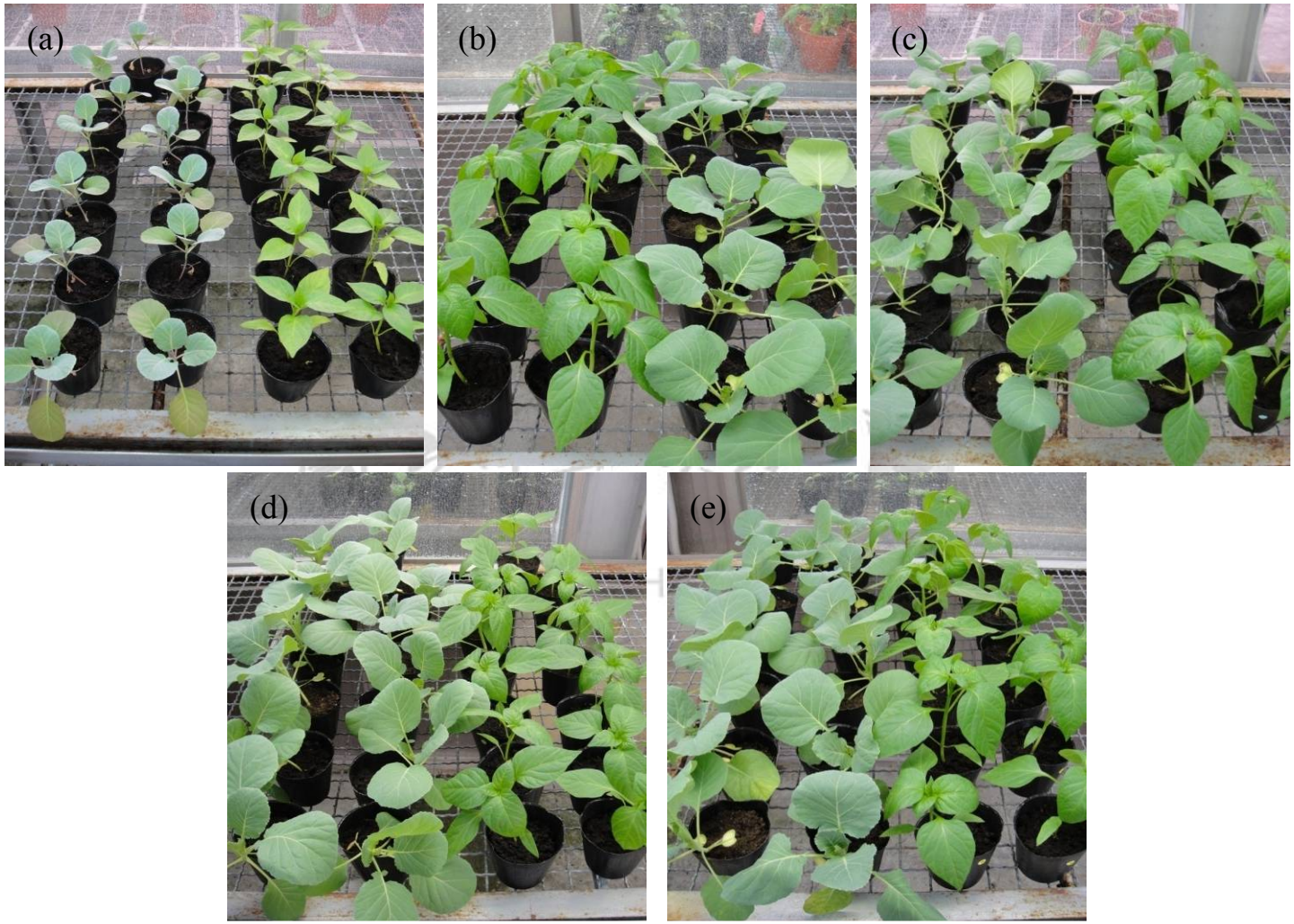

Figure 6. Plants treated with different SM treatments by soil mixture. (a) CK. (b) PCK. (c) CHT2401. (d) CHT2402. (e) CHR001. CK: water; PCK: soybean milk; CHT2401, CHT2402, and CHR001 mean different strains of *Bacillus mycoides* cultured in soybean milk.

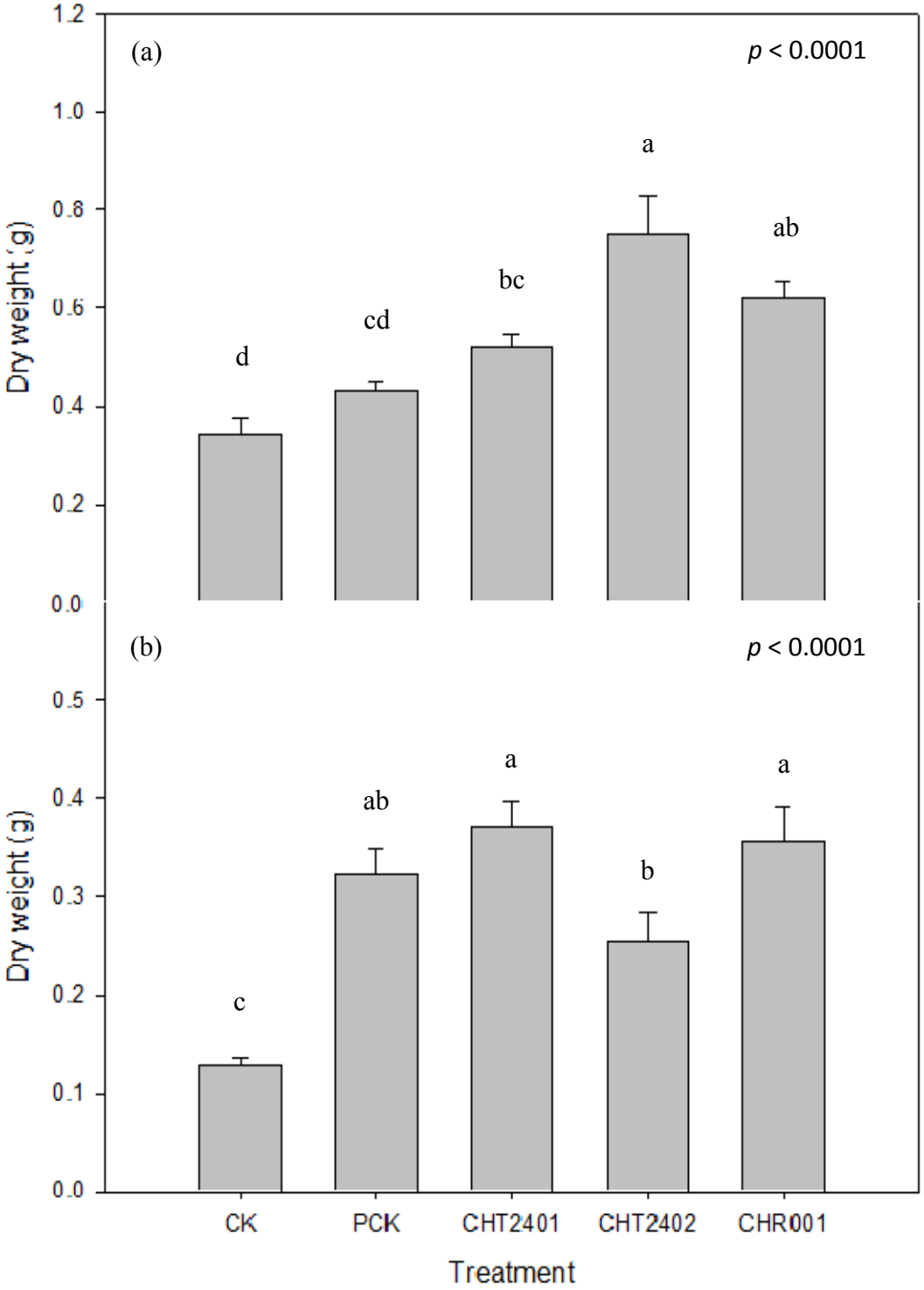

Figure 7. The dry weight of plants under different SM treatments by soil mixture. (a) cabbage. (b) sweet pepper. Bars without the same letters are significantly different ($p < 0.05$, Fisher's test). CK: water; PCK: soybean milk; CHT2401, CHT2402, and CHR001 mean different strains of *Bacillus mycoides* cultured in soybean milk.

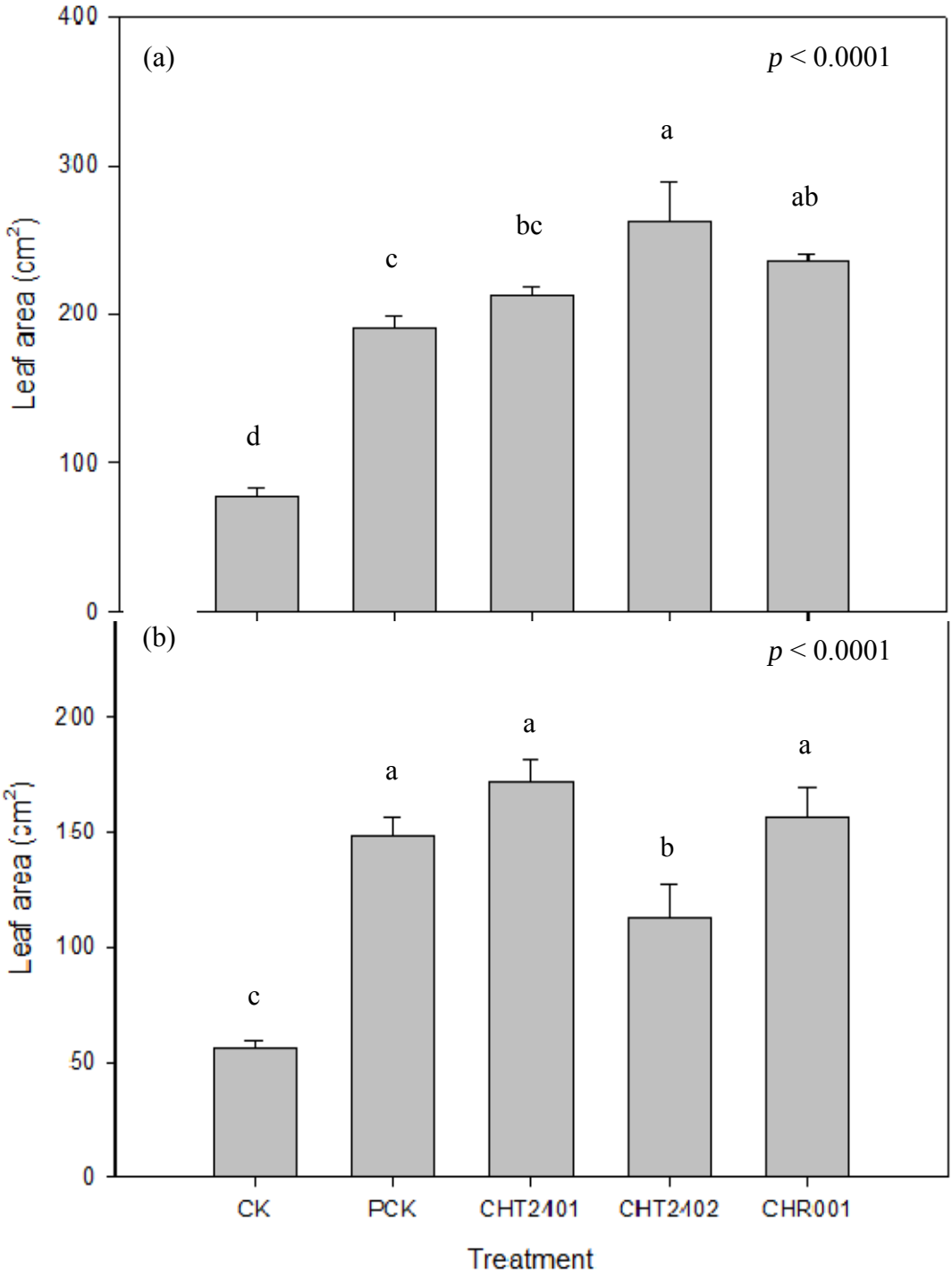

Figure 8. The leaf area of plants under different SM treatments by soil mixture. (a) cabbage. (b) sweet pepper. Bars without the same letters are significantly different ($p < 0.05$, Fisher's test). CK: water; PCK: soybean milk; CHT2401, CHT2402, and CHR001 mean different strains of *Bacillus mycoides* cultured in soybean milk.

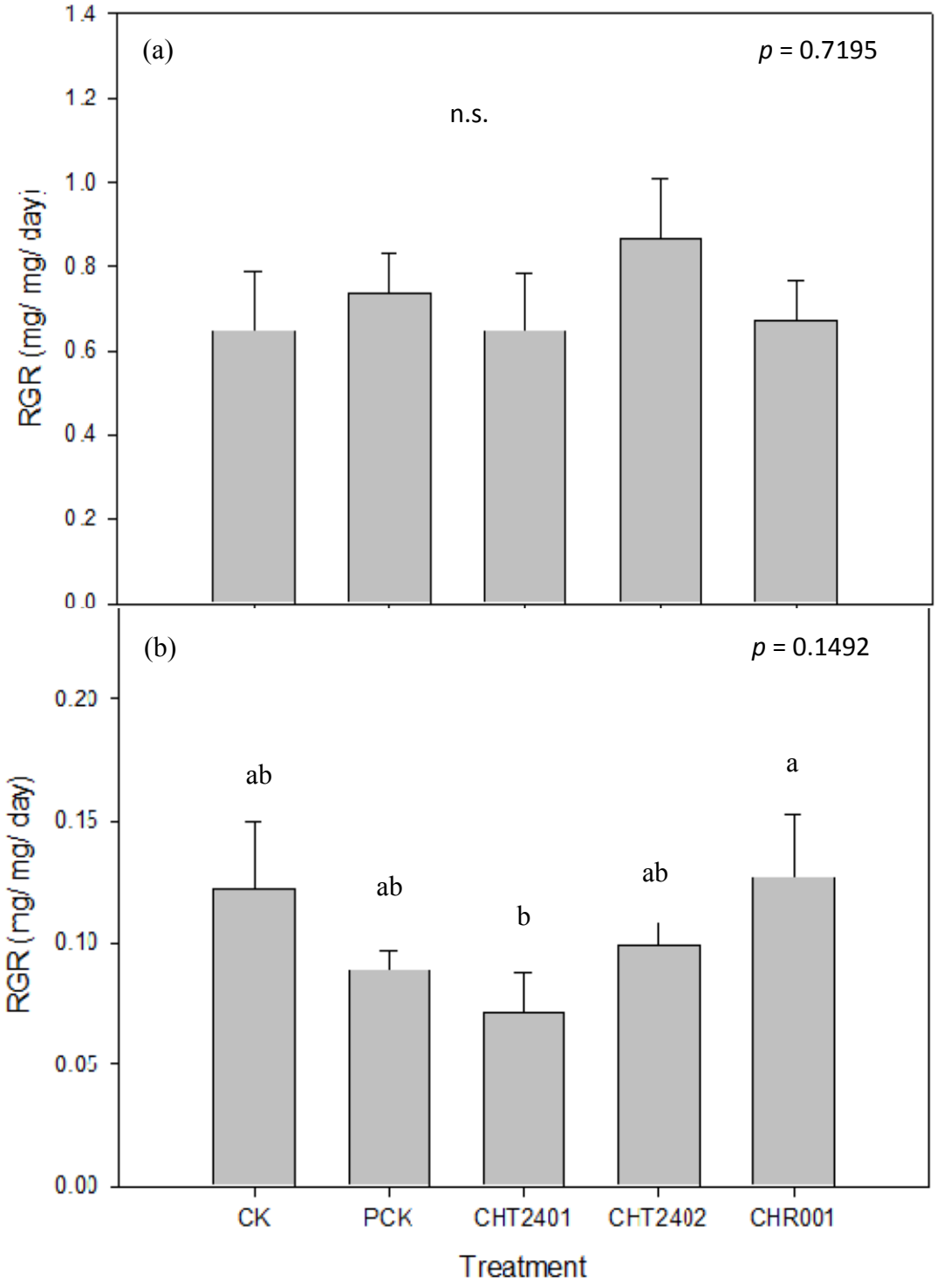

Figure 9. The RGR of tobacco cutworm larva when feeding on treated plants. (a) cabbage. (b) sweet pepper. Bars without the same letters are significantly different ($p < 0.05$, Fisher's test). CK: water; PCK: soybean milk; CHT2401, CHT2402, and CHR001 mean different strains of *Bacillus mycoides* cultured in soybean milk.

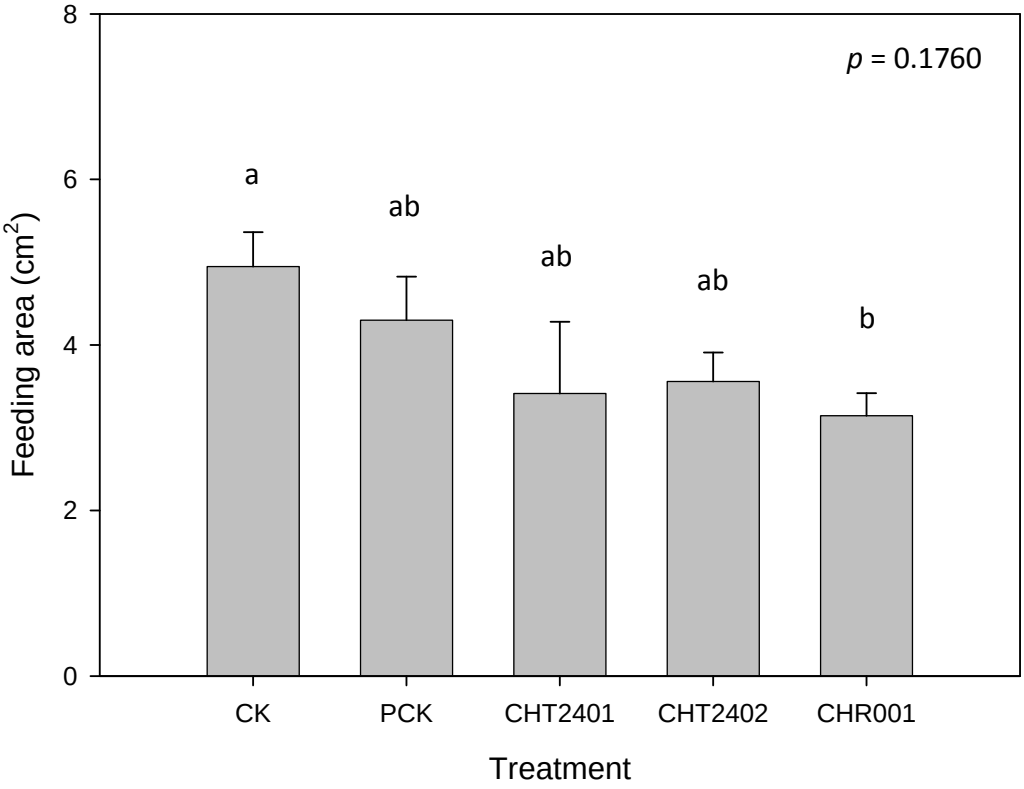

Figure 10. The feeding area of 4th instar tobacco cutworms when fed on treated cabbage for two days. Bars without the same letters are significantly different ($p < 0.05$, Fisher's test). CK: water; PCK: soybean milk; CHT2401, CHT2402, and CHR001 mean different strains of *Bacillus mycoides* cultured in soybean milk.

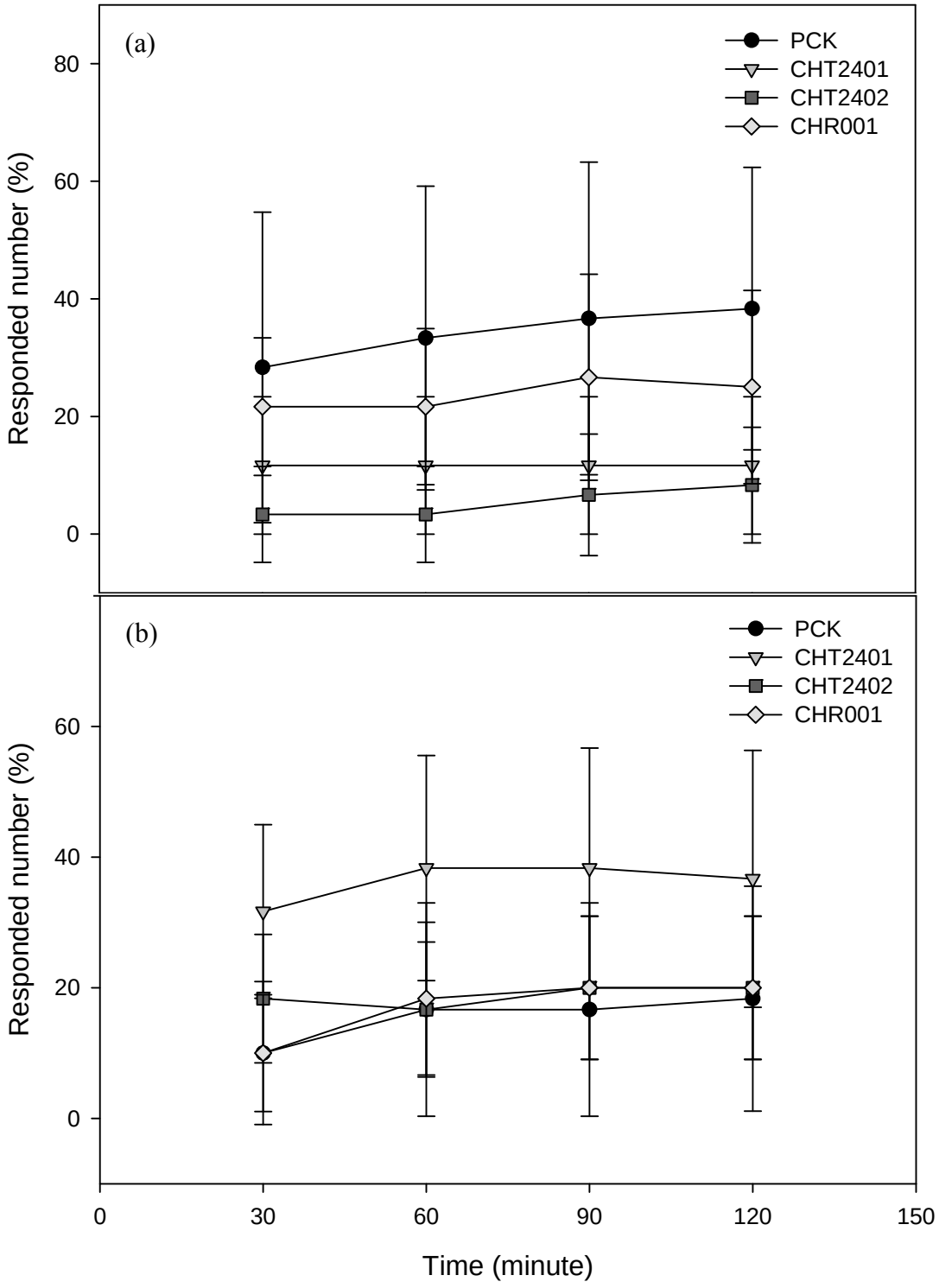

Figure 11. The responded number of cutworms to differently treated leaves without additional suspension spray. (a) cabbage. (b) sweet pepper. PCK: soybean milk; CHT2401, CHT2402, and CHR001 mean different strains of *Bacillus mycoides* cultured in soybean milk.

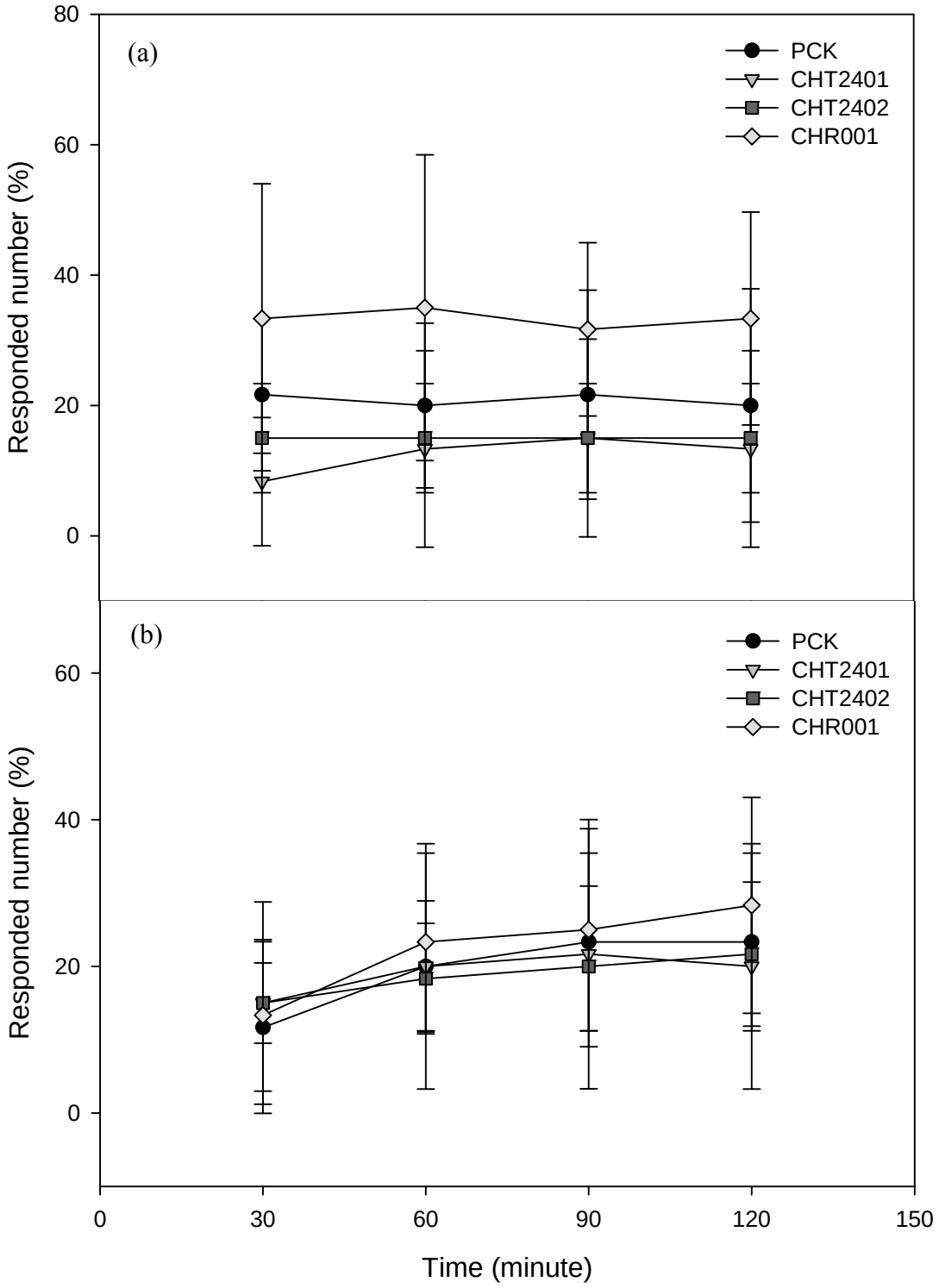

Figure 12. The responded number of cutworms to differently treated leaves with additional suspension spray. (a) cabbage. (b) sweet pepper. PCK: soybean milk; CHT2401, CHT2402, and CHR001 mean different strains of *Bacillus mycoides* cultured in soybean milk.

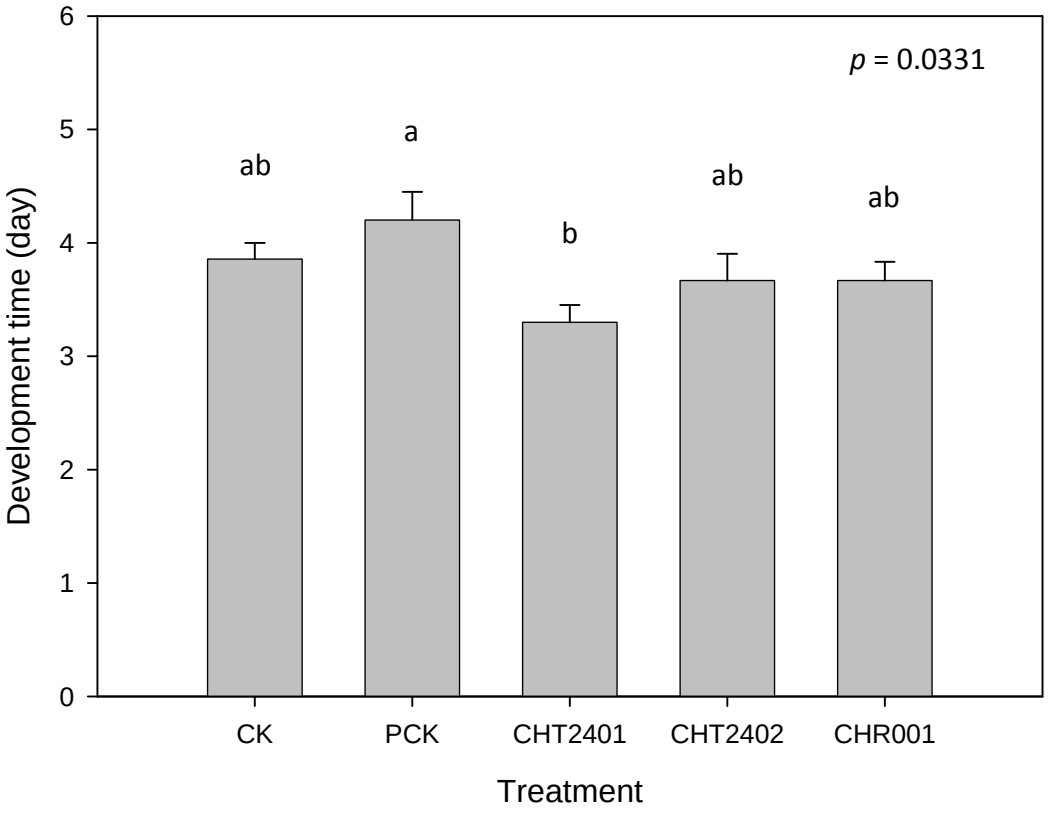

Figure 13. The development time of aphids from 4-day-old nymph to 1-day-old adult when feeding on treated cabbage. Bars without the same letters are significantly different ($p < 0.05$, Fisher's test). CK: water; PCK: soybean milk; CHT2401, CHT2402, and CHR001 mean different strains of *Bacillus mycoides* cultured in soybean milk.

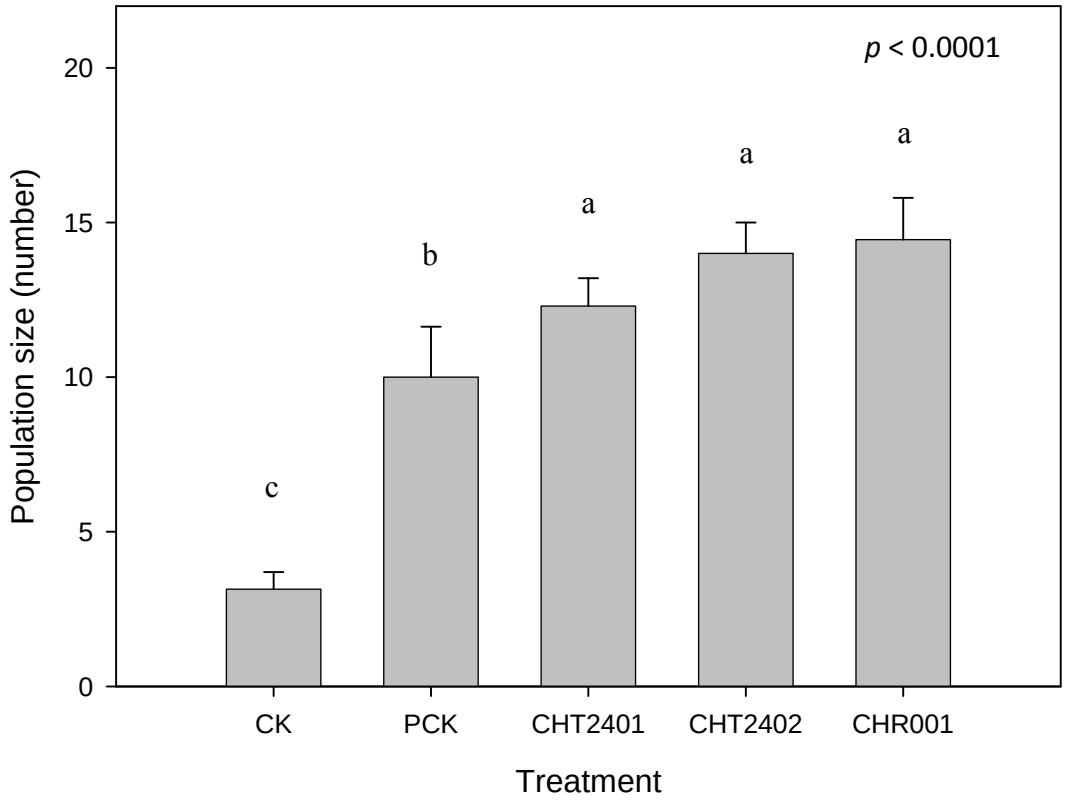

Figure 14. Effects of different treatments of cabbage on population size of aphids, 5 days after the first birth. Bars without the same letters are significantly different ($p < 0.05$, Fisher's test). CK: water; PCK: soybean milk; CHT2401, CHT2402, and CHR001 mean different strains of *Bacillus mycoides* cultured in soybean milk.

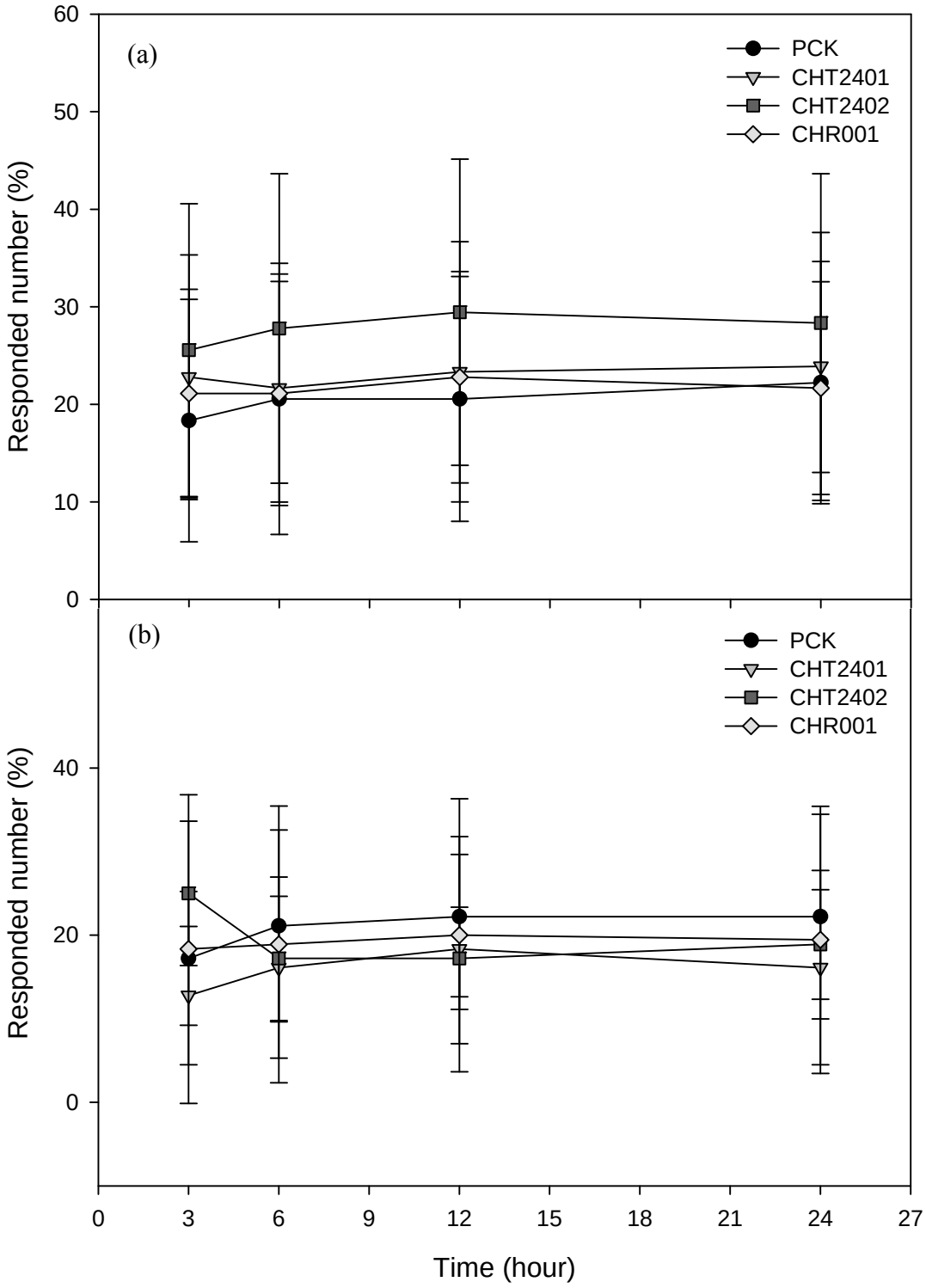

Figure 15. The responded number of aphids to differently treated leaves without additional suspension spray. (a) cabbage. (b) sweet pepper. PCK: soybean milk; CHT2401, CHT2402, and CHR001 mean different strains of *Bacillus mycoides* cultured in soybean milk.

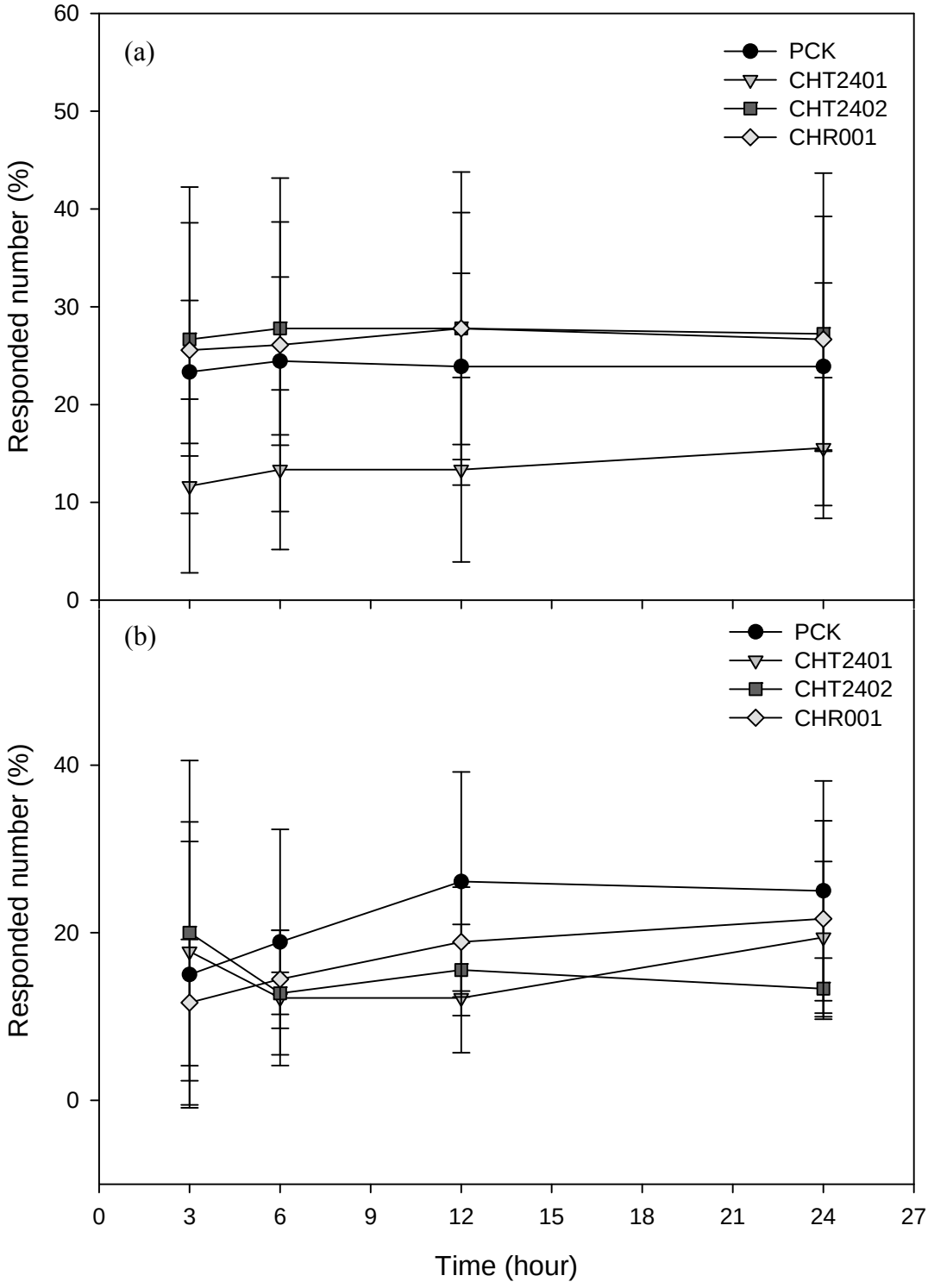

Figure 16. The responded number of aphids to differently treated leaves with additional suspension spray. (a) cabbage. (b) sweet pepper. PCK: soybean milk; CHT2401, CHT2402, and CHR001 mean different strains of *Bacillus mycoides* cultured in soybean milk.

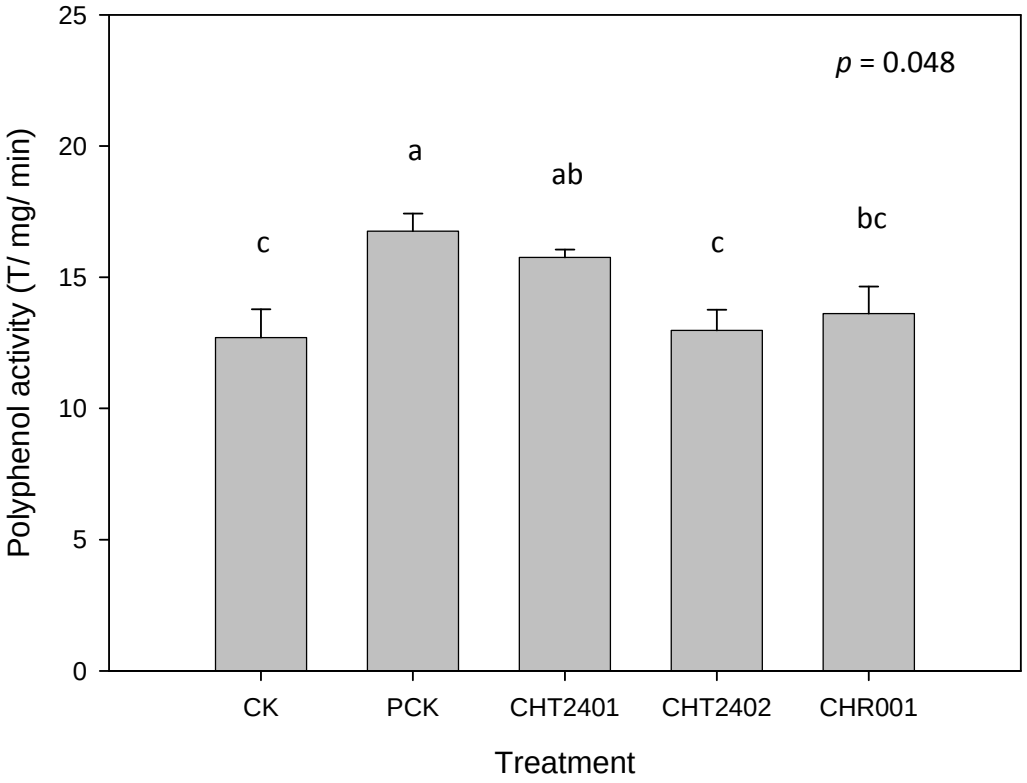

Figure 17. Polyphenol oxidase activity of cabbage when plants were 4-week-old without insect feeding. Bars without the same letters are significantly different ($p < 0.05$, Fisher's test). CK: water; PCK: soybean milk; CHT2401, CHT2402, and CHR001 mean different strains of *Bacillus mycoides* cultured in soybean milk.

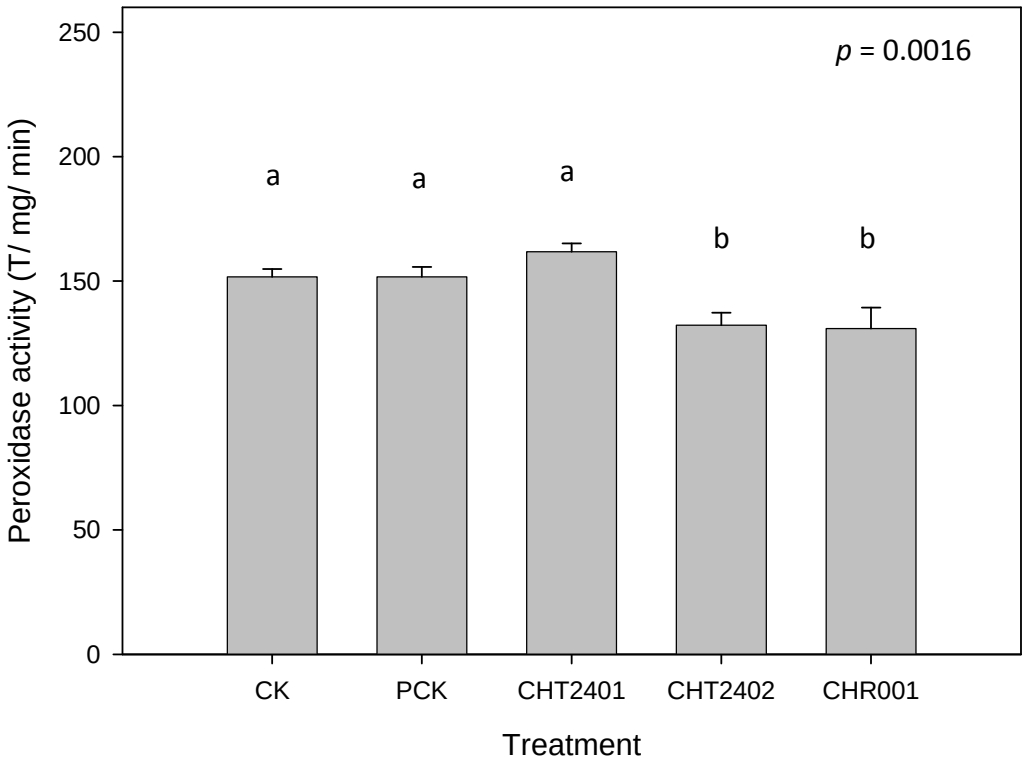

Figure 18. Peroxidase activity of cabbage when plants were 4-week-old without insect feeding. Bars without the same letters are significantly different ($p < 0.05$, Fisher's test). CK: water; PCK: soybean milk; CHT2401, CHT2402, and CHR001 mean different strains of *Bacillus mycoides* cultured in soybean milk.

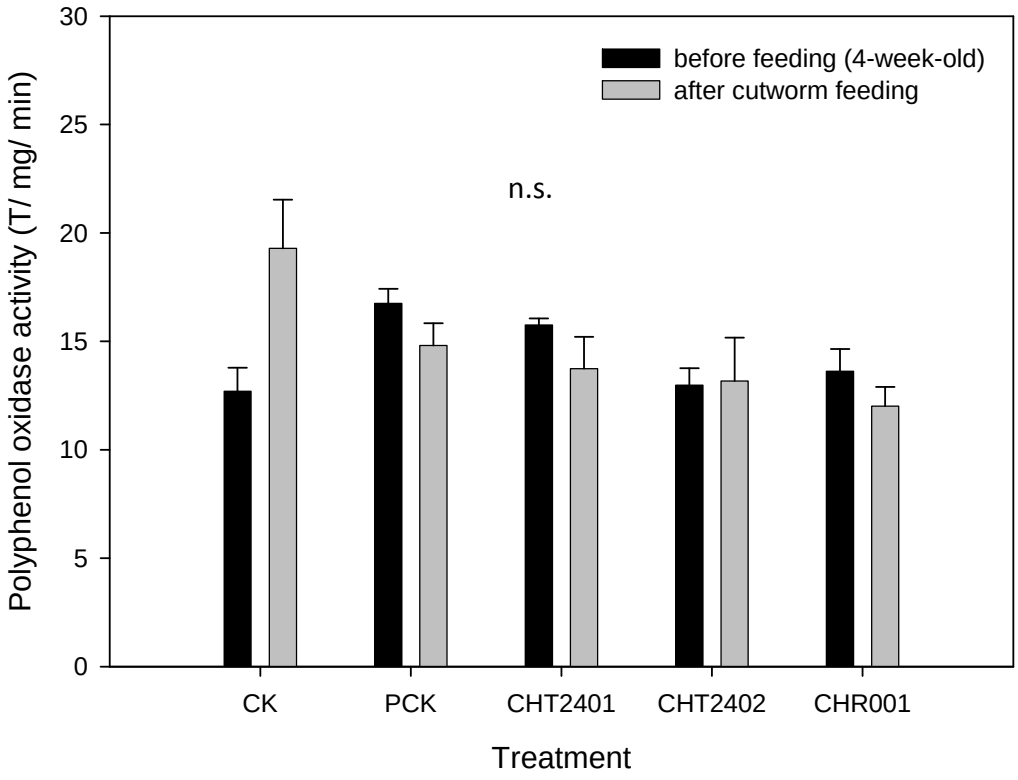

Figure 19. Polyphenol oxidase activity of cabbage when plants were 4-week-old and 4-week-old but treated with cutworm feeding. *, **, *** Significant at 5%, 1%, and 0.1% levels, respectively. ($p < 0.05$, Fisher's test). CK: water; PCK: soybean milk; CHT2401, CHT2402, and CHR001 mean different strains of *Bacillus mycoides* cultured in soybean milk.

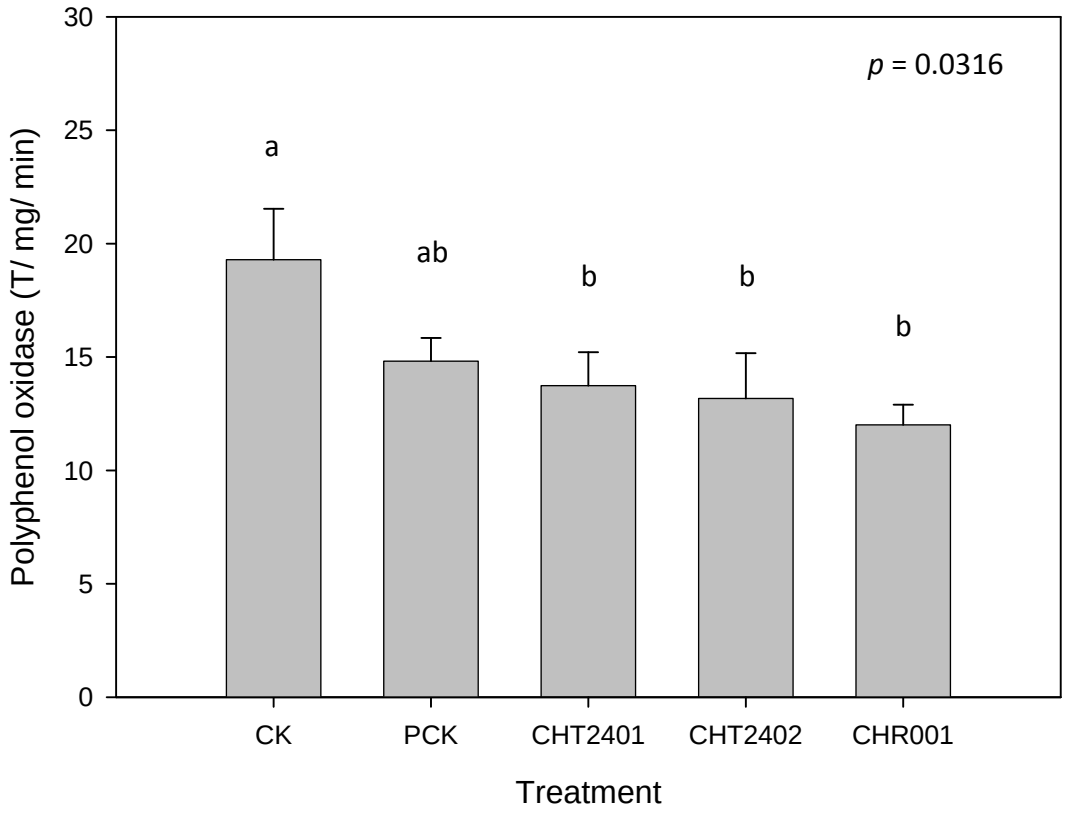

Figure 20. Polyphenol oxidase activity of cabbage when plants were 4-week-old and treated with cutworm feeding. Bars without the same letters are significantly different ($p < 0.05$, Fisher's test). CK: water; PCK: soybean milk; CHT2401, CHT2402, and CHR001 mean different strains of *Bacillus mycoides* cultured in soybean milk.

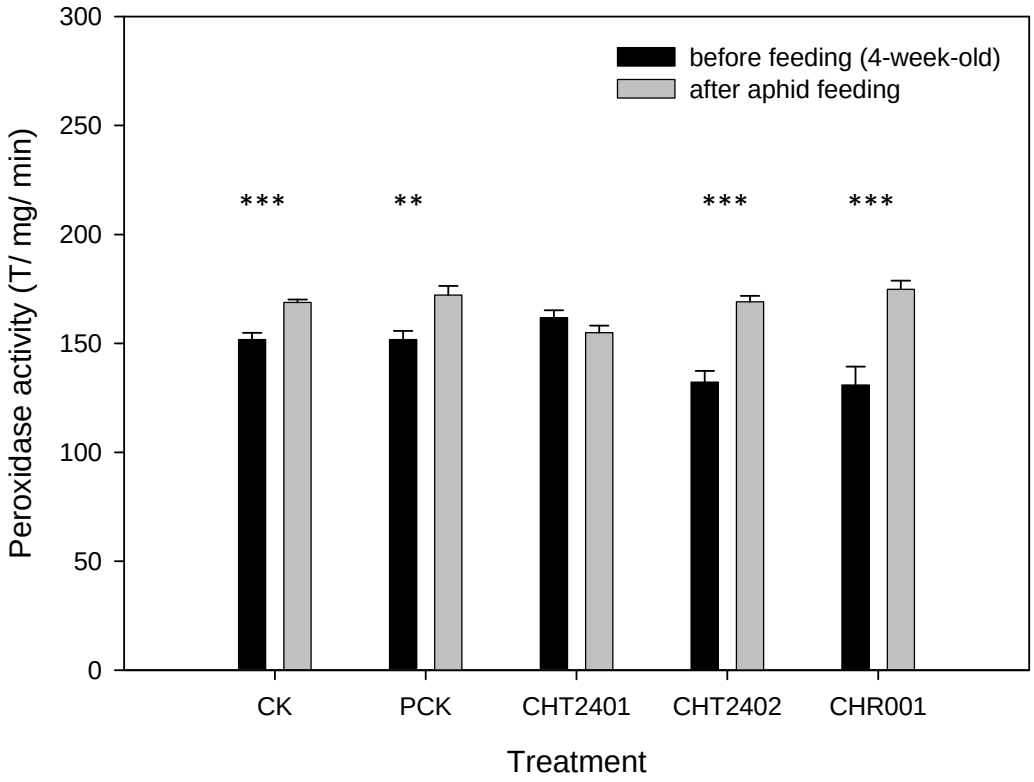

Figure 21. Peroxidase activity of cabbage when plants were 4-week-old and 4-week-old but treated with aphid feeding. *, **, *** Significant at 5%, 1%, and 0.1% levels, respectively. CK: water; PCK: soybean milk; CHT2401, CHT2402, and CHR001 mean different strains of *Bacillus mycoides* cultured in soybean milk.

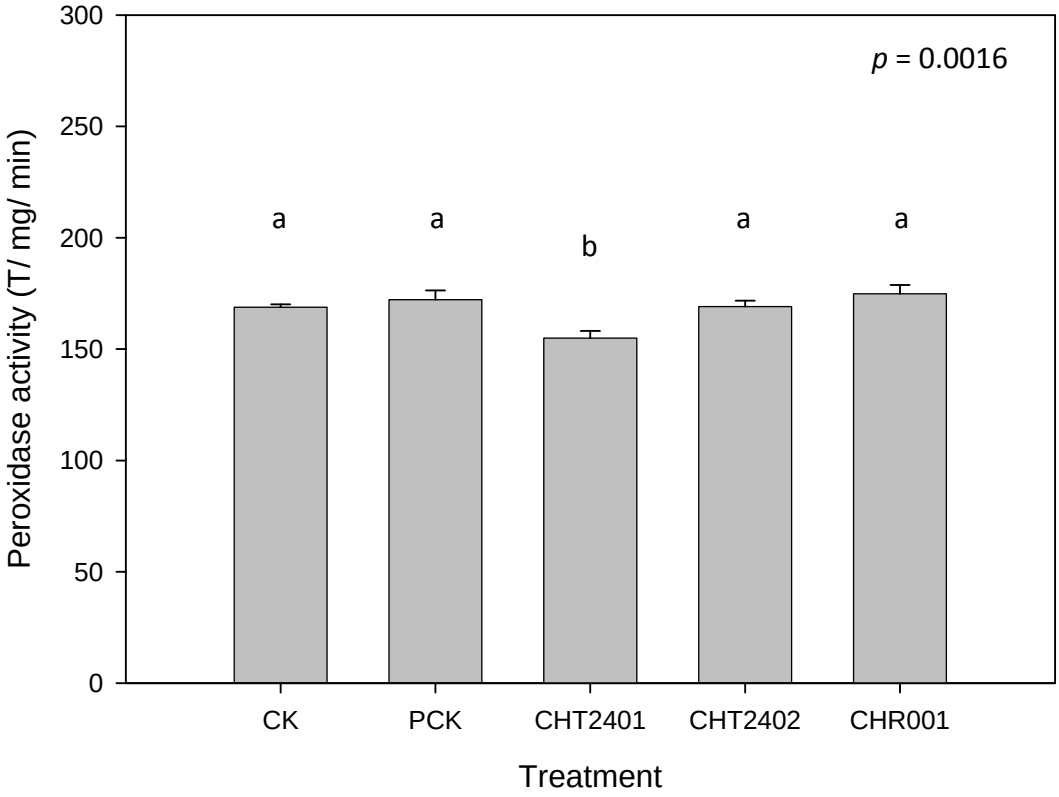

Figure 22. Peroxidase activity of cabbage when plants were 4-week-old and treated with aphid feeding. Bars without the same letters are significantly different ($p < 0.05$, Fisher's test). CK: water; PCK: soybean milk; CHT2401, CHT2402, and CHR001 mean different strains of *Bacillus mycoides* cultured in soybean milk.