Synergistic control against Asian corn borer (ACB) by *Trichogramma* vectored *Beauveria bassiana* infection on survival larvae escaped from parasitism

Yang Lu¹, Li Sui¹, Zhi Yang¹², Gang Mao¹, Wen-jing Xu¹, Yu Zhao¹, Le Li¹, 2, Qi-yun Li¹, * and Zheng-kun Zhang¹

¹Jilin Academy of Agricultural Sciences, Jilin Key Laboratory of Agricultural Microbiology, Key Laboratory of Integrated Pest Management on Crops in Northeast China, Ministry of Agriculture and Rural Affairs of the People’s Republic of China, Jilin, China

²College of Agricultural and Life Sciences, Jilin Agricultural University, Jilin, China

E-mail: qyli1225@126.com

**Abstract.** The combined application of natural enemies and entomopathogens as an alternative pest-control may act synergistically, additively or antagonistically. The objective of this study is to determine the efficacy of the combined application by parasitic *Trichogramma dendrolimi* (Hymenoptera: *Trichogrammatidae*) carrying insect-pathogenic fungus *Beauveria bassiana* (Bals.) Vuill. (TCB) against Asian corn borer (ACB), *Ostrinia furnacalis* (Lepidoptera: Pyralidae). 0.1% (w/v) starch solution was selected from several adjuvants due to its highest ability to carry fungal conidia, over $3.60 \times 10^4$ conidia per wasp. The fungal conidia adsorbed on the surface of factitious host, *Antheraea pernyi* eggs, subsequently adhered on new emerged *T. dendrolimi* as a vector, which carried the conidia onto eggs’ surface of ACB. The *B. bassiana* had no influence on *T. dendrolimi*’s parasitic rate, whereas resulting in fungal conidia adhering on over 60.00% of hatched larvae, which consequently caused 27.00 ± 5.70% percent of muscardine cadaver rate of pest’s larvae in a screen house test. In field trial, it showed significantly lower incidence parameters regarded as number of damaged plants, wormholes, and living pests, per 100 plants in TCB treatment compared to that of sole *T. dendrolimi* release (non-TCB) and non-treatment as control, respectively, especially the number of muscardine cadaver per 100 plants was 6-fold amount higher than that of non-TCB treatment. In this study, the suitable adjuvant of cost-effective and safe to *T. dendrolimi* emergence was selected for fungal conidia adhering for TCB, and the fungal conidia transmitting process from host eggs to pest infection was visualized by fluorescence observation, on the basis, it was proved in both screen house and field experiments that the TCB was a synergistic ACB control program with labor saving, low cost and high efficient advantage compared to sole biocontrol agent application.

1. Introduction

The lepidopteran Asian corn borer (ACB), *Ostrinia furnacalis* (Guenée), is a common borer pest throughout the Eastern China and Southeast Asia, as well as the Pacific and Australasia [1]. It is one of the most destructive pests of *Zea mays*, *Sorghum bicolor*, *Setaria italica* and *Gossypium spp.* in worldwide and usually causes annual production loss of 6 to 9 billion kg in China [2]. A strong
growth in use of biological control agents, is taking place in worldwide, due to its inherent positive characteristics such as healthier for farm workers and persons living in farming communities, no harvesting interval or waiting period after release of agents, sustainable as there is no development of resistance against arthropod natural enemies, no phytotoxic damage to plants, better yields and a healthier product, reduced pesticide residues[3].

There are approximately 650 species of this egg parasitoid genus, of which approximately 200 species are considered to attack the eggs of various crop pests, and approximately 70 species have been mass-produced and released against lepidopterous pests of different crops [4]. Trichogramma is a kind of egg parasitoids, which actively searched for their host and laid eggs in the host egg, and subsequently its larvae eat the egg yolk and cause the host to die before emergence to realize the pest management [4,5]. Trichogramma has been widely used in the biological control of lepidopteran pests for more than 80 years [4], and it has been released for control of corn borer at large scale for 50 years in China [2].

Beauveria bassiana (Bals.) Vuill. is one of the most known entomopathogenic fungal species, and has a wide host range of over 700 species of insects and arachnids, causing white muscardine disease [6,7]. Owing to its broad host range and is eco-friendly to the environment than chemical pesticides, B. bassiana was thought to be critical means for pest control [3,8]. Furthermore, B. bassiana employ a variety of insecticidal mechanisms, such as secreting insect cuticle-degrading enzymes, synthesizing toxic secondary metabolites, and invading and suppressing the insect immune system to avoid the possibility of the host developing resistance [9]. However, as living microorganisms, the biocontrol efficacy of fungal pesticide is intrinsically mediated by abiotic environmental factors, most noticeably humidity, temperature, rainfall, and solar radiation [10]. Its major formulations using in the field or greenhouse were wettable powder, granular formulations and water or soil-based suspension [11-13], so it needs a more efficiency and bioactive application measure in field to realize the cost-efficacy. Natural enemies as biocontrol agents have been large scale mass production and regular releases in worldwide, and is often a commercial activity [14].

Since the first report by Peng et al.[15] that pollinators vectors have been used in entomovectoring studies, bees such as honey bees [Apis mellifera Linnaeus (Hymenoptera: Apidae)], bumble bees [Bombus impatiens Cresson (Hymenoptera: Apidae)] and Bombus terrestris Linnaeus (Hymenoptera: Apidae) and in some particular cases the mason bee [Osmia cornuta Latreille (Hymenoptera: Megachilidae)] were used as vectors carried microbial control agents against pathogens were used for plant disease management [16]. And also, the pollinators carried microbial control agents such as entomopathogenic microorganisms (B. bassiana, M. anisopliae, and Bacillus thuringiensis) [17-19] and virus such as Heliothis armigera Nuclear Polyhedrosis Virus (HaNPV), have been widely applied for insect pest management [20]. However, in this strategy, it remains challenge of vector population and also allowing pollination to be synchronized with the blooming period of crops, in addition, the target of bees or pollinators was flower, the biocontrol agents carried by the bees could hardly arrive suffering parts and affect precisely. In recent years, the combination of various biocontrol agents with different insecticidal mechanisms could take advantages of different bio-defense resources to improve the efficiency of pest prevention and control, thus effectively solving the bottlenecks mentioned above. Mohammed and Hatcher [21] determined the most effective timing of application of fungi and parasitoids combination to control Myzus persicae (Sulzer) in laboratory and field. Rauch et al. [22] used a two-year field study by a blend of entomopathogenic nematode (Heterorhabditis bacteriophora) and entomopathogenic fungi (Metarhizium brunneum) in conjunction with chemical insecticides to determine the effect against western corn rootworm. Ramirez-Ahuja et al. [23] carried out laboratory experiments to investigate the combined effect of parasitoid and zoophytophagous bug for the potato-psyllid control. All above research hence suggested that the combination of the two biological control measures, especially the combined application of biocontrol agents with different mechanisms, was potential to improve the pest management effect. However, there was few of literatures on combined application of natural enemies and entomopathogenic microorganisms on pest control.
In this work, we established a method to enhance the biocontrol efficiency against ACB by *Trichogramma dendrolimi* (*Hymenoptera: Trichogrammatidae*) carrying *B. bassiana* (TCB), by making visualized transmission of one biocontrol agents by another using a fluorescence tracing method, on the basis of suitable adjuvant screening, and finally verified its cost-effective synergistic control against ACB in screen house and field.

2. Materials and methods

2.1. Fungal strain and cultivation

The *B. bassiana* strain, BbOFDH1-5, was isolated from muscardine cadavers of ACB in corn field (N44°31′23″, E125°51′59″), Dehui City, Jilin Province, China in 2008. It was isolated on the basis of morphological features [24], and verified by internal transcribed spacer region (ITS) sequencing analysis, using the universal primers ITS1 and ITS4[25]. The strain was deposited in Agriculture Culture Collection of China, and preservation number is ACCC No. 32726, and its LT50 against 2nd ACB larvae was described as Sui [26].

GFP labeled *B. bassiana* strain BbOFDH1-5-GFP was constructed by PEG mediated protoplast transformation [27]. The strain was deposited in China General Microbiological Culture Collection Center, and preservation number is CGMCC No. 15673.

The aerial conidia powder containing $1 \times 10^{11}$ conidia/g of the two strains determined under the microscope using a Neubauer hemocytometer were prepared by liquid-solid two-phase fermentation [28] and stored at 4°C for further uses. Conidial viability was tested for both strains by carrying out a germination test. A low concentration of conidia was suspended in 0.1 % Tween 80 solution and a 20 μl aliquot from this stock was plated on potato dextrose agar (PDA) medium. The aliquot was spread with a sterile glass rod onto the medium surface and incubated at 25℃ for 24 h. Conidia germination was checked under the microscope with description of Jaber [29] three randomly selected groups of 100 conidia were assessed for germination. Only conidia with a germ tube as long as the conidium widths were considered to have germinated. Germination exceeded 93 % for each strain on average.

2.2. Eggs of *Antheraea pernyi*

Cocoons of *A. pernyi* strain JL, were provided by Jinong high-tech development Co., Ltd, Gongzhuling, Jilin province, China, which originally collected from Jilin city, Jilin Province, China. The cocoons were stored at 4°C and moved to 25℃, 60% R.H. for emergence as needed for the experiments. The mated female moths were collected and confined in Lock & Lock plastic boxes (24.8 cm × 18.0 cm × 9.3 cm, L×W×H). One to three days later, the abdomen of the female moths was separated and the eggs were pressed out in a clean bench. After carefully washing with sterilized distilled water by one to two times, the mature eggs were spread out on sterile paper tissue at room temperature to dry. Eggs of *A. pernyi* were kept at 4°C for no more than 15 days until they were used in experiments.

2.3. Mass rearing of *T. dendrolimi*

Adult mated *T. dendrolimi* strain BC were provided by Jinong high-tech development Co., Ltd, Gongzhuling, Jilin province, China, which and collected from Jilin city, Jilin Province, China, with natural host was *Dendrolimus punctatus* WIK. The *A. pernyi* eggs were exposed to the parasitoids for 24 hr [30]. The parasitized eggs were placed in plastic bottles (10 cm in diameter x 7.0 cm in height) at 25℃, 60% R.H. until adult wasps emerged. The female wasps used for experiments were 12 to 24 h post emergence.

2.4. Eggs and larvae of ACB

Egg masses of the ACB were collected from corn fields (43.52° N, 124.79° E) in Gongzhuling, Jilin Province in China, in July 2017, and were reared in an environmental chamber maintained at (25 ± 1) ℃, with (70 - 80) % R.H. and 18L: 6D photocycle[31]. Newly hatched larvae were transferred to a plastic box (20 cm in diameter and 6 cm in height) with artificial diet developed by Guo *et al.*[32]. The larvae were kept in the plastic box for further experiments.
2.5. Maize seedling
Maize seeds (cv. Jidan 558) were surface-sterilized with 1% sodium hypochlorite for 2 min, followed by 2 min in 70% ethanol, rinsed in sterile water three times. Rinse the seeds three times in sterile distilled water. In screen house experiment, surface sterilized seeds were planted in seedling pots (30 cm diameter × 40 cm high) filled with sterilized compost, garden soil and vermiculure at 5:4:1 ratio. Soil mixes were sterilized twice in an autoclave for 2 h at 121°C at each sterilization time, giving a 24 h interval between each sterilization and allowing soil to cool for 72 h before use and fertilized using 1 g L⁻¹ Basfoliar 30-10-10+Mg+TE (COMPO Expert GmbH, Krefeld, Germany) inorganic fertilizer and adjusted to a concentration of 1 ×10⁵ conidia per milliliter, the fungal conidia germination rate was checked according to above mentioned method post incubation for 24 hours under room temperature.

The three kinds of adjuvants and sterile water as control were used for wetting the surface of A. pernyi eggs by spaying, and which was put into fungal conidia powder for adhering, sterile water (H₂O) was used for control. Ten A. pernyi eggs have been parasitized for 11 days of all treatments and control (with no B. bassiana conidia adhering) were put into in plastic Petri dishes (90 mm) for incubation at 26°C, 60% R.H., and 14 L:10 D photoperiod. All treatments were performed for 5 times. The wasp’s emergence was observed at 0 h, 6 h, 12 h, and 24 h, respectively. The conidia number adhered the surface of ten randomly selected survival wasps in each treatment was calculated by blood cell count under microscope. All above A. pernyi eggs after emergence were dissected, and counted the amount of unemerged T. dendrolimi pupas to calculated emergence rate by the formula u-T/ (u-T + T) × 100% , in which u-T was the number of unemerged T. dendrolimi and T was the number of emerged T.

2.7. Fluorescence observation
The GFP-labeled B. bassiana strain BbOFDH1-5-GFP conidia were used for tracing observation on A. pernyi eggs, wasps and larvae body surface of ACB to indicate the infection process of B. bassiana against the pest by its localization and distribution. Suitable adjuvant of 0.1% Starch soluble solution was used according to the results mentioned above, the treatment of A. pernyi eggs adhering conidia of B. bassiana were repeated, with non-adhering control, and were incubated at 26°C, 60% R.H., and 14 L:10 D photoperiod. All treatments were performed for 10 replicates, five wasps were selected randomly from every replicate for fluorescence observation. During whorl stage of maize, small pieces of corn leaves with egg masses spawned by female adults of ACB post 24-48 hours of mating were cut off, and put into dishes, A. pernyi eggs after mating, treated by B. bassiana conidia adhering and only selected adjuvant as control, a female wasp emerged from the A. pernyi eggs of treatment and control was randomly selected based on morphological characteristics of female genitalia[33] and released into dish with above corn leave, respectively, all treatments were performed for 10 times and were observed at 24 h after wasps release. The parasitized egg masses were incubated at 26°C, 60% R.H., and 14 L:10 D photoperiod, all treatments were performed for 10 replicates. Ten surviving larvae from each replicate were selected randomly for observation after hatching, and calculated the fungal conidia adhering rate.
The confocal images of all samples were collected by single channel scans with a 488 laser for GFP fluorescence under the 10 × objective using a Leica TCS SP8X laser scanning confocal microscope.

2.8. Screen house experiment for egg parasitism and larvae infection of pest
A high bioactivity against ACB B. bassiana strain BbOFDH1-5 conidia was used for ACB control in screen house experiment. Every five screen houses (5.0 m×5.0 m×3.5 m, L × W × H, 60 mesh) were respectively used for TCB, no fungal conidia carrying of T. dendrolimi (non-TCB), and non-release of T. dendrolimi (Control). Ten maize seedlings were planted in every screen house, which was 50 meters distance for each other. Ten female wasps that emerged from fungal carrying and non-carrying hosts eggs after mating in host eggs were randomly selected by morphological observation and released into screen house with above 50 ACB egg masses randomly distributed, which were spawned by female adults of corn borer after mating. Five parasitised egg masses were randomly selected from every screen house at about 24 h after TCB or non-TCB release, the eggs number of every mass was counted and recorded under the microscope, and the hatched larvae of ACB from parasitised egg mass was collected and counted. parasitic rate and corrected parasitic rate were calculated, parasitic rate was (1-(number of larvae / number of eggs)) × 100% in every replicate, and the corrected parasitic rate was parasitic rate of TCB and non-TCB × Average parasitism rate of control. All survival larvae were reared in sterile 1.5-ml Eppendorf tubes singly, the muscardine cadaver rate was calculated at 7 d after rearing.

2.9. Field trial
A corn field trial was conducted at Lishu city, Jilin province of China (N 43°21′, E 124°09′) in 2018. Three trials were set for TCB release, non-TCB release, and non-release against ACB, respectively, every treatment was performed in five field, which was one thousand square meters at 500 meters distance distributed randomly, One hundred surface wet A. perryi eggs mixed with 0.25 g 1×10^{11} conidia/g BbOFDH1-5 powder, and put the eggs into plastic wasps release-ball (China Patent Number ZL 2017 2 0555354.1), which was water-resistant for releasing T. dendrolimi in field, the fungal conidia amount was checked ensuring above 3 × 10^{4} conidia/wasp carrying. Two hundred balls were used evenly in every treatment for first TCB or non-TCB release at June 20, 2018, when the average number of pest egg masses/100 plants was 1-1.5 in the corn field by investigation according to Wang et al.[2], and the same for the second release was at June 25, 2018. five days after the first release, both the two days were not overcast and rainy. The number of damaged plants, wormholes, living larvae, and muscardine cadavers per 100 plants were investigated considered as parameters to identify the control efficiency of TCB compared to that of non-TCB release only and non-release control before harvest, respectively, in which the damaged plants were regarded as having symptoms of wormhole, stem and male ear broken on whole maize.

2.10 Statistical analysis
Data were tested for normality assumptions using qqplot before analysis. Levene’s homogeneity test and Shapiro-Wilk normality test were set at the 0.05 significance level. We used one-way ANOVA to test the difference in the conidia carrying capacity by three adjuvants. We also used one-way ANOVA to test the effects of adjuvants and B. bassiana on the emergence time, survival amount, the emergence rate of wasp, and the control efficiency against Asian corn borer of TCB in both screen house and field experiments. Mean values were compared by Duncan’s Multiple Range Test (P < 0.05). All statistical analyses were performed using SPSS 17.0 (SPSS Inc., 2008).

3. Results
3.1. Effects of adjuvants and B. bassiana on Trichogramma
All tested adjuvants showed no influence on B. bassiana conidia germination, which were all between 93% to 97%. As shown in figure 1A, in all treatments the T. dendrolimi began to emerge from host eggs within 16 hours to 24 hours and reached the peak at 24 hours post inoculation. All wasps emerged completely at 24 hours post inoculation, and the average emergence amount of each egg was 50 to 60, and there was no significant difference among all treatments (F = 1.252, P < 0.05). After
emergence, *Trichogramma* wasps began to die, and reached zero survival at about 72 hours post inoculation in all treatments. Survival numbers of *Trichogramma* wasps in each group were observed at 48 h and 72 h, respectively. Notably, there was no significant difference in the number of living *Trichogramma* wasps between 48 h ($F = 1.624, P < 0.05$) and 72 h ($F = 0.37, P < 0.05$) after emergence. The results of emergence rate ($F = 0.987, P < 0.05$) were mentioned in figure 1B. The *Trichogramma* wasp emergence rate and survival amount of each treatment was between 70% and 80%, demonstrating no difference. The spore germination rate of *B. bassiana* was not affected by these additives.

![Figure 1](image1.png)

**Figure 1.** Effect of *B. bassiana* and adjuvants on *Trichogramma*. a, the effect of different adjuvants and *B. bassiana* on the emergence time and survival amount of *T. dendrolimi*; b, the emergence rate of *T. dendrolimi* in different treatments. “n.s.” indicates no significant differences among treatments at $p = 0.05$ (LSD after repeated-measures One-way ANOVA).

As shown in figure 2, the *B. bassiana* conidia carrying capacity of *Trichogramma* wasps gave significant difference in different treatments with H$_2$O, PDS, SS and Glu as adjuvants, while the fungal conidia carrying capacity of *Trichogramma* wasps reached $10^4$ orders of magnitude in all treatments. Among them, the conidia carrying capacity of wasp in SS and Glu treatments was more than $3.56 \times 10^4$ conidia/wasp, which was significantly higher than the other two treatments.

![Figure 2](image2.png)

**Figure 2.** The amount of *B. bassiana* conidia carrying on *T. dendrolimi* body in different treatments. For treatments by different adjuvants, mean ($\pm$SE) amount of fungal conidia followed by different letters within the same sampling date indicate significant differences among treatments at $p = 0.05$ (LSD after repeated-measures one-way ANOVA).
3.2. Transmission of fungal conidia from A. pernyi eggs to ACB larvae

For all samples in control group, low background fluorescence were detected (figure 3F), whereas fluorescence of conidia in treatment group could be observed on the whole insects’ body (figure 3G), especially on head (figure 3H), abdomen (figure 3H and figure 3J), wings (figure 3I) and legs (figure 3J). After parasitism, the surface of pest eggs in TCB and non-TCB treatment was observed. As shown in figure 4, there was no fluorescence was observed on all the collected pest eggs surface from non-TCB treatment (figure 4E), in contrast, the fluorescence conidia distribution could be observed in that of TCB treatment (figure 4F). For the survival larvae, there was no fluorescence conidia distribution existed on the pest bodies surface came from non-TCB treatment (figure 4G), while there were still obviously fluorescence conidia distribution on that came from TCB treatment (figure 4H).

![Figure 3](image1.png)

**Figure 3.** Fluorescence observation of *T. dendrolimi* and *T. dendrolimi* carrying spores of *B. bassiana*. (A) The image of *T. dendrolimi* by bright field, the scale bar = 100 μm; (B) The image of TCB, the scale bar = 100 μm; (C) The image of TCB’s head and legs, the scale bar = 75 μm; (D) The image of TCB’s wing, the scale bar = 75 μm; (E) The image of TCB’s abdomen and legs, the scale bar = 75 μm; (F) The background fluorescence image of *T. dendrolimi*, the scale bar = 100 μm; (G) The fluorescence image of TCB, the scale bar = 100 μm; (H) The fluorescence image of TCB’s head and legs, the scale bar = 75 μm; (I) The fluorescence image of TCB’s wing, the scale bar = 75 μm; (J) The fluorescence image of TCB’s abdomen and legs, the scale bar = 75 μm.

![Figure 4](image2.png)

**Figure 4** Fluorescence observation on the surface of corn borer eggs and body surface of corn borer larva after parasitized by *T. dendrolimi* carrying spores of *B. bassiana*. (A) The image of the control eggs; (B) The image of the treatment eggs; (C) The image of the control body surface; (D) The image of the treatment body surface; (E) The fluorescence image of the control eggs; (F) The fluorescence image of the treatment eggs; (G) The fluorescence image of the control body surface; (H) The fluorescence image of the treatment body surface. The scale bar = 75 μm.
3.3. ACB larvae escaped from parasitism were infected by TCB in screen house experiment

Not all pest eggs were parasitized by *T. dendrolimi* (figure 5A), the corrected parasitism rate of TCB and non-TCB were 50.19% and 51.70%, respectively, with no significant difference. For the survival larvae, there was 27.00 ± 5.70% of muscardine cadaver rate of TCB treatment, whereas no muscardine cadaver existed in all larvae from non-TCB treatment and non-release control 7 d after larvae collection (figure 5B). It should be noted that in all TCB treated green houses, there were large amount of muscardine cadavers of young larval stage existed on the surface of maize plants (Fig. 5B), while the remaining larvae after collection infected by fungal conidia of TCB, they did not bore the plants.

![Figure 5. The control efficiency against Asian corn borer of TCB under the condition of screen house. The gray bars represent the parasitic rate of eggs of Asian corn borer, and the dark bars represent the muscardine cadaver rate after treated by *T. dendrolimi* carrying of *B. bassiana*. Bars (±SE) with “*” within the same assessment date indicate significant differences among treatments, and “n.s.” indicates no significant differences among treatments at *p* = 0.05 (LSD after repeated-measures One-way ANOVA).](image)

### 3.4. Biocontrol efficiency of TCB against ACB in field

As shown in table 1, the number of damaged plants, wormholes, and alive pests of *T. dendrolimi* release only were 45.64, 56.46, and 29.44 per 100 plants, respectively, which were significantly lower than that of control, which were 86.82, 170.45 and 67.83, respectively. In other words, in these three aspects, the TCB treatment compared to the former were 47.43%, 66.88% and 56.60% decline, respectively, displayed a much higher control efficiency. Compared to *T. dendrolimi* release only, the control efficiency of number of damaged plants, wormholes, and alive pests increased by 28.1%, 22.8%, and 24.5% after TCB treatment. For muscardine cadaver, no significant difference was achieved between *T. dendrolimi* release only treatment and control, which were only 1.44 and 1.82, respectively, while it was 9.41 per 100 plants in TCB treatment which demonstrated a higher efficiency.

| Table 1. The control efficiency against Asian corn borer of TCB in field. |
|---------------------------------------------------------------|
| Control | Non-TCB | TCB |
| Number of damaged plants per 100 plants | 86.82±3.50 | 45.64±5.54 | 21.24±3.60 |
| Number of wormholes per 100 plants | 170.45±10.73 | 56.46±5.72 | 17.65±3.50 |
| Number of alive pest larvae per 100 plants | 67.83±3.93 | 29.44±2.89 | 12.81±1.43 |
| Number of muscardine cadaver per 100 plants | 1.82±0.58 | 1.44±0.75 | 9.41±0.74 |

For different treatments and control, mean (±SE) number of damaged plants, wormholes, alive pest larvae and muscardine cadaver per 100 plants, followed by different letters within the same sampling
date indicate significant differences among treatments at $p = 0.05$ (LSD after repeated-measures one-way ANOVA).

4. Discussion

Natural enemies and microbial organisms are used in large numbers to reduce pests, because they are healthier for farm workers and persons living in farming communities, easy to realize professionalism, saving agricultural production when pesticides fail or are not available, reduced and replaced the synthetic pesticides, and resolved insecticide resistance problem [3,34]. However, there are some inherent shortcomings in biocontrol agents on the market, the parasitic natural enemies only work on the eggs of pests with about 60 percent parasitism rate, but have no control effect on larval stage, that leading to a wormhole damage on plants by survival larvae escaped from parasitism. Additionally, the entomopathogenic fungi can only infect larvae of insect pests, but have no effect on eggs. Thus, the entomopathogenic microorganisms carried by natural enemies to realize attractive pest management measure was considered as a good solution [35,36]. In present study, the two worldwide used biocontrol agents *T. dendrolimi* and *B. bassiana* have different mechanism were combined and applied against ACB, lead to a significantly enhance on control efficiency against the pest compared to that of sole *T. dendrolimi* application by making cooperative use of their mechanism on pest control.

The popular application mode of *T. dendrolimi* was *A. pernyi* eggs as an factitious host [37] uniformly distributed on cardboard, and all emerged wasps into field from one hole for pest control. When the *A. pernyi* eggs exposed in nature, the environmental factors just like ultraviolet rays and rainwater influenced the control efficiency of emerged wasps. For *B. bassiana*, Aerial conidia are the primary infective propagule of this entomopathogens, its hydrophobic asexual spore is relatively easy and inexpensive for mass-production [12], while he hydrophobicity of *B. bassiana* aerial conidia was a key problem for its formulation and exploitation. Commercial *Beauveria* products can be divided into dry and liquid formulations, which usually contains compatible adjuvants and other necessary additives to form a stable preparation. Its application was limited by many natural and environmental factors and shelf-life [2,3]. The major application methods of *B. bassiana* were similar to chemical pesticides by granule spreading and inundative sprays, resulting in most of active conidia distributed on leaves, surface of plants and ground, while the pest eggs and larvae were on back of plant leaves, so few of fungal conidia could adhere on that and reduce the contact and infection opportunity, and as the larvae growing, it became more and more difficult to be pathogenic for entomopathogens against the pests. As forementioned, bees were used as vectors for *B. bassiana* conidia transmission, which was applied in greenhouse for pests control successfully, in which the fungal spores was are assigned to the bees’s body in a special customized wooden dispenser[18,19,38]. It is convenient for greenhouse or limited field applications, while it is not satisfactory for field crops. Here, a kind of plastic wasps release-ball contained about one hundred of *A. pernyi* eggs was used, it could realize the fully mixed of *A. pernyi* eggs in a confined space with little amount of fungal conidia about 0.70 g/hm², which could be stored under suitable conditions to guarantee their bioactivity for a long time, concurrently, the measure could protect the both agents against environmental impact, and also, it was suitable for UAV precisely application in filed to reduce labor force.

A high virulent strains BbOFDH1-5 of *B. bassiana* was used in this study for field control against ACB by TCB upon screening of adjuvants employed for fungal conidia adhering on host eggs, all tested adjuvants showed no negative influence on fungal conidia germination as well as the emergence and survival of *T. dendrolimi*. It has been proved that there was no negative effect on the regards such as emergence time, sex ratio, longevity, and parasitism of adult wasps of *T. pretiosum* treated by *B. bassiana*, by Potrich et al. [39,40]. Furthermore, there was no influence of *B. bassiana* on parasitism rate of *T. dendrolimi* which was verified in screen house experiment. It hence is viable and safe for pest control using *T. dendrolimi* and *B. bassiana* cooperatively. The 0.1% (w/v) starch solution was suitable because of its significant high conidia carrying ability, which could take more conidia onto the surface of pest eggs, and give more adhering chance for pest larvae to realize the infection. In addition,
the starch is a kind of food and industrial material suitable for large scale application for TCB, because it is low cost and easy to produce.

5. Conclusion
In present study, the fungal conidia were transmitted to the surface of pest eggs precisely via a highly efficient vector of parasitic insect, by which the newly hatched larvae can be infected during the process of their crawling and egg feeding after hatching, to realize the continuous control in different growth stages of pests, it is necessary to clear the transmission process from parasitic insects to pests. Green fluorescent protein (GFP) labeling is an effective tracker to study the gene function, colonization, interaction for visible tracing of animals, plants, and microorganisms [27,41,42]. In the research, it gave us an intuitive observation the process and distribution of fungal conidia transmission from host eggs to *Trichogramma* wasp, pest eggs and finally pest larvae, and proved the activity of TCB against ACB was a continuous process.

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References
[1] Nafus D M and Schreiner I H 1991 Review of the biology and control of the Asian corn borer, *Ostrinia furnacalis* (Lep: Pyralidae) *International Journal of Pest Management*, 37 41–56
[2] Wang Z Y, He K L, Zhang F, Lu X, and Babendreier D 2014 Mass rearing and release of *Trichogramma* for biological control of insect pests of corn in China *Biological Control* 68 136–44
[3] van Lenteren J C, Bolckmans K, Köhl J, Ravensberg W J and Urbanèja A 2018 Biological control using invertebrates and microorganisms: plenty of new opportunities *BioControl* 63 39–59
[4] Mills N 2009 Egg parasitoids in biological control and integrated pest management. *Egg parasitoids in agroecosystems with emphasis on Trichogramma*, eds Consoli F L, Parra J R and Zucchi R A (Springer, Dordrecht) pp. 389–11
[5] Burcham D C, Abarrientos Jr N V, Wong J Y, Ali M I M, Fong, Y K and Schwarze F W 2017 Field evaluation of *Trichoderma* spp. as a biological control agent to prevent wood decay on Benin mahogany (*Khaya grandifoliola*) and rain tree (*Samanea saman*) in Singapore *Biological Control* 114 114–24
[6] Faria M R and Wraight S P 2007 Mycoinsecticides and Mycoacaricides: A comprehensive list with worldwide coverage and international classification of formulation types *Biological Control* 43 237–56
[7] McKinnon A C, Saari S, Moran-Diez M E, Meyling N V, Raad M and Glare T R 2017 *Beauveria bassiana* as an endophyte: a critical review on associated methodology and biocontrol potential *BioControl* 62 1–17
[8] Vega F E, Posada F, Aime M C, Pava-Ripoll M, Infante F and Rehner S A 2008 Entomopathogenic fungal endophytes *Biological Control* 46 72–82
[9] Leger R J S and Wang C S 2010 Genetic engineering of fungal biocontrol agents to achieve greater efficacy against insect pests *Applied Microbiology and Biotechnology* 85 901–7
[10] Fernandes É K K, Rangel D E N, Braga G U L and Roberts D W 2015 Tolerance of entomopathogenic fungi to ultraviolet radiation: a review on screening of strains and their formulation *Current Genetics* 61 427–40
[11] Coombes C A, Hill M P, Moore S D and Dames J F 2016 Entomopathogenic fungi as control agents of *Thaumatotibia leucotreta* in citrus orchards: field efficacy and persistence *BioControl* 61 729–39
[12] Mascarín G M and Jaronski S T 2016 The production and uses of Beauveria bassiana as a microbial insecticide World Journal of Microbiology and Biotechnology 32 177

[13] Rice S J, Baker D K and Leemon D M 2019 Development of mycoinsecticide formulations with Beauveria bassiana and Metarhizium anisopliae for the control of lesser mealworm, Alphitobius diaperinus, in chicken broiler houses BioControl 64 489–500

[14] van Lenteren J C 2012 The state of commercial augmentative biological control: plenty of natural enemies, but a frustrating lack of uptake BioControl 57 1–20

[15] Peng G, Sutton J C and Kevan P G 1992 Effectiveness of honeybees for applying the biocontrol agent Gliocladium rosea to strawberry flowers to suppress Botrytis cinerea Canadian Journal of Plant Pathology 14 117–29

[16] Hokkanen H M T and Menzler-Hokkanen I 2007 Use of honeybees in the biological control of plant diseases Entomological Research 37 62–63

[17] Jyoti J L and Brewer G J 1999 Honey bees (Hymenoptera: Apidae) as vectors of Bacillus thuringiensis for control of banded sunflower moth (Lepidoptera: Tortricidae) Environmental Entomology 28 1172–76

[18] Al-mazra’awi M S, Shipp L, Broadbent A B and Kevan P G 2006 Dissemination of Beauveria bassiana by honey bees (Hymenoptera: Apidae) for control of tarnished plant bug (Hemiptera: Miridae) on canola Environmental Entomology 35 1569–77

[19] Al-mazra’awi M S, Shipp L, Broadbent B and Kevan P G 2006 Biological control of Lygus lineolaris (Hemiptera: Miridae) and Frankliniella occidentalis (Thysanoptera: Thripidae) by Bombus impatiens (Hymenoptera: Apidae) vectored Beauveria bassiana in greenhouse sweet pepper Biological Control 37(1) 89–97

[20] Butt T M, Carreck N L, Ibrahim L and Williams I H 1998 Honey–bee–mediated infection of pollen beetle (Meligethes aeneus Fab) by the insect pathogenic fungus, Metarhizium anisopliae Biocontrol Science and Technology 8 533–38

[21] Mohammed A A and Hatcher P E 2017 Combining entomopathogenic fungi and parasitoids to control the green peach aphid Myzus persicae Biological Control 110 44–55

[22] Rauch H, Steinwender B M, Mayerhofer J, Sigsgaard L, Eilenberg J and Enkerli J 2017 Field efficacy of Heterorhabditis bacteriophora (Nematoda: Heterorhabditidae), Metarhizium brunneum (Hypocreales: Clavicipitaceae), and chemical insecticide combinations for Diabrotica virgifera virgifera larval management Biological Control 107 1–10

[23] Ramirez-Ahuja M dL, Rodríguez-Leyva E, Lomeli-Flores J R, Torres-Ruiz A and Guzmán-Franco A W 2017 Evaluating combined use of a parasitoid and a zoophytophagous bug for biological control of the potato psyllid, Bactericera cockerelli Biological Control 106 9–15

[24] Humber R A 1997 Manual of Techniques in Insect Pathology, ed. Lacey L A (London: Academic Press) chapter 5 pp. 153–85

[25] White T J 1990 Analysis of phylogenetic relationships by amplification and direct sequencing of ribosomal RNA genes. PCR Protocols: A Guide to Methods and Applications, ed. White T J, Bruns T, Lee S J W T, Taylor J, Innis M A and Gelfand D H (New York: Academic) pp. 315–22

[26] Sui L, Zhu H, Xu W J, Guo Q F, Wang L and Zhang Z K 2020 Elevated air temperature shifts the interactions between plants and endophytic fungal entomopathogens in an agroecosystem Fungal Ecology 47 (10) 100940

[27] Ying S H and Feng M G 2006 Novel blastospore-based transformation system for integration of phosphinothricin resistance and green fluorescence protein genes into Beauveria bassiana Applied Microbiology and Biotechnology 72 206–10

[28] Ying F M, Chen Q C, Ma H T, Zhang S H, Li Z M and Chen J W 1996 Study on standard massproduction of Beauveria bassiana Journal of Anhui Agricultural University 3 313–20

[29] Jaber L R 2018 Seed inoculation with endophytic fungal entomopathogens promotes plant growth and reduces crown and root rot (CRR) caused by Fusarium culmorum in wheat Planta 248 1525–35
[30] Lü X, Han S C, Li L Y, Grenier S and De Clercq P 2013 The potential of trehalose to replace insect hemolymph in artificial media for Trichogramma dendrolimi (Hymenoptera: Trichogrammatidae) *Insect Science* **20** 629–636

[31] Zhao C and Li Q 1996 Control of sex pheromone biosynthetic pathway by PBAN in Asian corn borer, Ostrinia furnacalis *Insect Science* **3** 354–67

[32] Guo L, Zeng X Y, Wang D Y and Li G Q 2010 Methanol metabolism in the Asian corn borer, Ostrinia furnacalis (Guenée) (Lepidoptera: Pyralidae) *Journal of Insect Physiology* **56** 260–65

[33] Ma C S and Chen Y W 2006 Effects of constant temperature, exposure period, and age on diapause induction in Trichogramma dendrolimi *Biological Control* **36** 267–73

[34] Otieno J A, Pallmann P and Poehling H M 2017 Additive and synergistic interactions amongst Orius laevigatus (Heteroptera: Anthocoridae), entomopathogens and azadirachtin for controlling western flower thrips (Thysanoptera: Thripidae) *BioControl* **62** 85–95

[35] Jacobson R J, Chandler D, Fenlon J and Russell KM 2001 Compatibility of Beauveria bassiana (Balsamo) Vuillemin with Amblyseius cucumeris Oudemans (Acarina: Phytoseiidae) to control Frankliniella occidentalis Pergande (Thysanoptera: Thripidae) on cucumber plants *Biocontrol Science and Technology* **11** 391–400

[36] Ikegawa Y, Ezoe H and Namba T 2015 Adaptive defense of pests and switching predation can improve biological control by multiple natural enemies *Population Ecology* **57** 381–95

[37] Liu S S, Zhang G M and Zhang F 1998 Factors influencing parasitism of Trichogramma dendrolimi eggs of the Asian corn borer, Ostrinia furnacalis *BioControl* **43** 273–87

[38] Kapongo J P, Shipp L, Kevan P and Broadbent B 2008 Optimal concentration of Beauveria bassiana vectored by bumble bees in relation to pest and bee mortality in greenhouse tomato and sweet pepper *BioControl* **53** 797–812

[39] Potrich M, et al. 2009 Seletividade de Beauveria bassiana e Metarhizium anisopliae a Trichogramma pretiosum Riley (Hymenoptera: Trichogrammatidae) *Neotropical Entomology* **38** 822–26

[40] Potrich M, Alves L F A, Lozano E, Roman J C, Pietrowski V and Neves P M 2015 Interactions between Beauveria bassiana and Trichogramma pretiosum under laboratory conditions *Entomologia Experimentalis et Applicata* **154** 213–21

[41] Errampalli D, Leung K, Cassidy M B, Kostrzynska M, Blears M, Lee H and Trevors J T 1999 Applications of the green fluorescent protein as a molecular marker in environmental microorganisms *Journal of Microbiological Methods* **35** 187–99

[42] Jin K et al. 2008 An improved method for Beauveria bassiana transformation using phosphinothricin acetyltransferase and green fluorescent protein fusion gene as a selectable and visible marker *Biotechnology Letters* **30** 1379–83