Effects of Bt-Cry1Ah1 Transgenic Poplar on Target and Non-Target Pests and Their Parasitic Natural Enemy in Field and Laboratory Trials

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Abstract: Increasing areas of artificial afforestation and poplar monoculture in China have led to serious problems with insect pests. The development of genetic engineering technology, such as transgenic modification with Bacillus thuringiensis (Bt) genes, provides novel solutions to the pest problem. We generated a Bt-Cry1Ah1 gene incorporating codon optimization and transferred it into Populus deltoides × P. euramericana cv “Nanlin895” using an Agrobacterium-mediated method. The resulting Bt-Cry1Ah1 transgenic poplars were planted in the field with permission from the State Forestry Administration in 2017. Field and laboratory studies were conducted in Jiangsu, China, to investigate the effects of these transgenic poplars expressing the Cry1Ah1 protein on target and non-target pests and their parasitic natural enemy. Target pests included Hyphantria cunea (Lepidoptera, Arctiidae), Micromelalopha troglodyta (Lepidoptera, Notodontidae), and Clostera anachoreta (Lepidoptera, Notodontidae). Plagiodera versicolora (Coleoptera, Chrysomelidae) served as the non-target pest. Laboratory trials showed that the six transgenic poplar lines exhibited resistance against the target insects. The corrected mortality rates of the target pest larvae fed leaves from the six lines were as high as 87.0%, significantly higher than that of the control. However, the corrected mortality rate of the non-target pest larvae was markedly lower and did not differ significantly from that of the control. Field experiments showed that transgenic poplar exhibited resistance against H. cunea and M. troglodyta. Field mortality rates were slightly higher than laboratory mortality rates. In addition, we investigated Choisoiia cunea (Hymenoptera, Eulophidae) as a parasitoid of H. cunea pupae that had been fed transgenic poplar leaves. The emergence time, parasitism rate, and abundance of C. cunea did not differ significantly from those of the control. Therefore, Bt-Cry1Ah1 transgenic poplar can be used to effectively control damage by target insect pests without negatively affecting non-target insects and parasitoids.

Keywords: Cry1Ah1; field and laboratory trials; transgenic poplar; target pest; non-target pest; natural enemies

1. Introduction

Poplar (Populus spp.) is one of the most widely distributed and adaptable forest genera in the world. It is important economically and ecologically, and plays an important role in the global ecosystem. However, due to single-species afforestation in large areas, poplar insect pests often occur, resulting in damage to poplar growth. These insect pests have become the main obstacle to the rapid development of the poplar industry [1,2]. In poplar plantations, insect damage is often caused by commonly occurring lepidopterans [3,4]. Lepidopteran pests, especially Hyphantria cunea (Lepidoptera,
Arctiidae) and Micromelalopha troglodyta (Lepidoptera, Notodontidae), cause the most damage every year in Jiangsu, China. Globally, H. cunea is considered a quarantine pest that harms many kinds of agricultural and forestry plants. It is listed in the first invasive alien species in China and is a target of interest in domestic forestry plant quarantine. This pest has caused great economic losses in China’s agricultural and forestry production, damaging the ecological stability of these two industries to a certain extent [5]. M. troglodyta is mainly distributed in northern and southern China. It is the main leaf-eating pest in poplar plantations. Numbers of M. troglodyta often increase rapidly under conditions of drought, high temperature, and other abnormal weather. The larvae of M. troglodyta feed on poplar leaves, leaving only the veins, which seriously affects photosynthesis and impacts poplar growth. In addition to the above two pests, Clostera anachoreta (Lepidoptera, Notodontidae) also seriously damages poplar trees in Jiangsu. It is considered one of the main pests of poplar and is mostly distributed in central and northern China. Additionally, this species is widely distributed and highly fecund.

Bt-Cry is one of the most widely used insect-resistance genes in the world. It is isolated from Bacillus thuringiensis (Bt) and exhibits high specificity [6,7]. So far, Bt-Cry has been used to effectively control insect pests in many plants including poplar, maize, rice, and cotton [8]. With the advancement in genetic engineering technology, new ways for improving insect-resistant poplar varieties are being developed [9]. Parsons et al. (1986) confirmed that poplar can express foreign genes through transformation technology [10]. Since then, various insect-resistance genes have been transferred to genetically engineered poplar trees, and effective pest control has been achieved. Tian et al. (1993) transferred a specific Bt gene that exerted a toxic effect on lepidopterans into Populus nigra var. italica and obtained good insect resistance [11]. Zheng et al. (2000) also created Bt-Cry1Ac transgenic poplar [12]. To date, Bt has been widely used to control insect damage in poplar. To improve insect resistance, various new methods have been used to produce transgenic insect-resistant poplar. These include the transfer of fusion genes and multiple genes as well as different transformation methods [13–16].

Transgenic plants have brought great benefits and convenience to human beings, but the safety of transgenic plants has attracted increasing attention [17,18]. The potential impact of transgenic plants on the ecology of natural systems has become a contentious issue and constitutes a major obstacle in the promotion of Bt transgenic plants. Many studies have reported on the ecological security of Bt transgenic plants, mainly focusing on the effects of transgenic plants on non-target insects and arthropods [19–21]. Recent studies have assessed the impacts of transgenic plants on non-target insects, microbial communities, and arthropod communities. For instance, Zhang et al. (2011) reported that Bt-Cry3A transgenic poplar did not significantly affect the mortality, exuviation index, pupation rate, and eclosion rate of the non-target insect C. anachoreta. Furthermore, the arthropod communities in Bt transgenic poplar and control field stands were similar, as indicated by four diversity indices (Berge–Parker, Shannon–Wiener, evenness, and Simpson’s inverted indices) and the Bray–Curtis index. Therefore, Bt-Cry3A poplar had no effects on a non-target pest (C. anachoreta) and generally did not have any significant negative effects on the poplar arthropod community [22]. Zuo et al. (2018) reported that 5-year-old transgenic poplars did not affect the stability of the arthropod community, nor the physical and chemical properties of the soil and soil microbial community structure [23]. Guo et al. (2016) discovered that there were no significant differences between transgenic and control maize in terms of diversity indices associated with natural enemies (Shannon–Wiener, Simpson’s, and Pielou’s indices) and their abundance. Furthermore, Bt maize did not exert any time-dependent effects on the entire arthropod natural enemy community, and no community dissimilarities were observed between Bt and non-Bt maize plots [24]. Because of the long poplar life cycle and the complex interactions between poplar and other organisms, there are many concerns about the risks transgenic poplars bring into the forest ecosystem. Although the field cultivation of transgenic poplar is restricted in many countries, there are few reports on poplar field trials. China is the first country to commercialize Bt transgenic poplar, and now it has the largest transgenic poplar plantations in the world. As such, the potential
ecological risks associated with these plantations have attracted much attention [25]. Therefore, it is necessary to assess the ecological risks associated with Bt transgenic poplar.

In this study, we screened for Cry1Ah protein and generated a Bt-Cry1Ah1 sequence that was codon optimized using Codon Poplar software (http://120.79.60.226:8080/u/chen/w/codonpoplar) based on the optimal codon usage of poplar [26]. Then, Bt-Cry1Ah1 was transferred into “Nanlin895” using an Agrobacterium-mediated method. The resulting transgenic poplars were planted in Jiangsu in 2017 after permission was obtained from the State Forestry Administration. In 2019, we examined the field-grown transgenic poplars to test for insect resistance and impacts on non-target insects and a parasitic natural enemy. The findings contribute to biosafety evaluations of Bt-Cry1Ah1 transgenic poplars and provide detailed guidance and a theoretical basis for the commercialization of Bt transgenic poplars.

2. Materials and Methods

2.1. Field Trials and Plant Materials

Six lines of Bt-Cry1Ah1 transgenic “Nanlin895” poplar (A-4-6, A-5-0, A-3-4, A-5-23, Z-1-3, and X-2-0), and non-transgenic wild type poplar (control, referred to as CK) were planted in Jiangsu in 2017 after permission was obtained from the State Forestry Administration. The trees were planted in lines of 121 trees each (12 rows and 12 columns) in a field with 2.0 m intervals between trees.

2.2. Enzyme-Linked Immunosorbent Assay (ELISA) Analysis of Cry1Ah1 Expression

Fresh and mature branches were collected (the third to sixth leaves) from transgenic and non-transgenic “Nanlin895” poplar when the branches were fully expanded in September 2019. The samples were placed in an icebox, taken back to the laboratory, and stored at −80 °C. A 0.1 g mixed sample was assayed to detect the presence of the toxin protein using an ELISA kit (EnviroLogix, Portland, ME, USA).

2.3. Effects of Bt-Cry1Ah1 Transgenic Poplar on Target Pests

Pupae of H. cunea, M. troglodyta, and Clostera anachoreta (Lepidoptera, Notodontidae) were collected from poplar trees in the field in Jiangsu in 2019 and hatched under laboratory conditions at 27 ± 2 °C. Subsequently, eggs were collected from mated female adults (Figure 1A–C). The larvae eggs were hatched and cultured for 1 day before the experiment. Healthy 1-day-old larvae were used for the feeding experiment. The larvae were fed with fresh, clean, fully expanded leaves collected from transgenic and CK poplar every 2 days. For each experiment, 15 larvae were used per treatment (five insects in one box, three boxes per treatment), and the experiment was repeated three times; thus, a total of 45 larvae were assayed per treatment. The larvae were cultured in a constant-temperature incubator set to 27 ± 2 °C under a 14-h light/10-h dark cycle (Figure 1E). Dead larvae were counted and removed each day. Larval mortality in H. cunea was counted on days 6, 12, and 18. Larval mortality in M. troglodyta and C. anachoreta was counted on days 6 and 12, and larval pupation was observed 3 days later.

The field-cage method was used to study transgenic poplar in the field, with poplar resistance to H. cunea and M. troglodyta measured under field conditions. A dense nylon net with a length of 50 cm and a width of 25 cm was placed around the top part of each transgenic poplar, and the upper and lower openings of the net were closed after inoculation (Figure 1D). At each treatment period, each tree received 15 first-instar larvae, with a total of 45 larvae used after the experiment was repeated three times. Larval mortality in H. cunea and M. troglodyta was calculated on days 18 and 12, respectively, as follows:

\[
\text{Corrected mortality} = \frac{\text{observed mortality} - \text{control mortality}}{1 - \text{control mortality}} \times 100\%
\]

\[
\text{Pupation rate} = \frac{\text{pupal number}}{\text{larval number}} \times 100\%
\]
Figure 1. Experiments with target insects. (A) Acquisition of *H. cunea*; a: mating, b: bagging, c: oviposition, d: pupation, e: larva, f: adult. (B) Acquisition of *M. troglodyta*; a: mating, b: bagging, c: oviposition, d: pupation, e: larva, f: adult. (C) Acquisition of *C. anachoreta*; a: mating, b: bagging, c: oviposition, d: pupation, e: larva, f: adult. (D) Field-cage testing; a: field insemination, b: field cage. (E) Experimental procedure for testing insect resistance; a: transgenic poplar, b: cleaning collected leaves, c: insect inoculation, d: feeding in incubator.

2.4. Effects of Bt-Cry1Ah1 Transgenic Poplar on a Non-Target Pest

*P. versicolora* is one of the pests of poplar and can be found feeding on poplar trees in Jiangsu. Here, *P. versicolora* eggs were collected in the field and incubated in the laboratory (Figure 2). The leaves of transgenic and non-transgenic poplars were used to feed the larvae, similar to the method used for the feeding experiments with *H. cunea* and *M. troglodyta*. Larval mortality was observed on day 10.

Figure 2. Diagram of *P. versicolora*; (A) eggs, (B) larvae, (C) adult.

2.5. Effects of Bt-Cry1Ah1 Transgenic Poplar on a Parasitoid Wasp

*Chouioia cunea* (Hymenoptera, Eulophidae) is the dominant natural enemy of pupal *H. cunea* and is characterized by a high parasitism rate, strong flight ability, strong fecundity, and high reproduction rate [27] (Figure 3A). First, *H. cunea* larvae were fed leaves of transgenic and non-transgenic poplar
until they pupated. The pupae were then stored in a refrigerator at 3 °C to slow down their further development. Parasitism was carried out after 1 week. For each experiment, five pupae were parasitized in each treatment. The experiment was repeated three times. Pupae were parasitized in glass tubes in a dark environment with the temperature controlled at 23 ± 2 °C and a wasp-to-pupae ratio of 1:10. After 3 days, the pupae were removed from the dark environment and placed in a constant-temperature incubator set to 23 ± 2 °C (Figure 3B). To obtain parasitoid individuals, Antheraea pernyi (Lepidoptera, Saturniidae) pupae were selected and placed in a conical flask at room temperature. After C. cunea individuals emerged, they were collected and packed separately. We managed to collect sufficient wasps in one pupation round. Parasitoid emergence rate was calculated as follows:

Parasitoid emergence rate = number of pupae from which parasitoids emerged/total number of pupae

Figure 3. The experiment of parasitic natural enemy. (A) C. cunea. a,b: A. pernyi pupa parasitized by C. cunea. c,d: Pupa of H. cunea parasitized by C. cunea. (B) Procedure of the parasitoid experiment; a: tussah pupa, b: C. cunea production, c: sub-package, d,e: parasitism process, f: sub-package.

2.6. Statistical Analysis

All experiments were performed in triplicate. Tukey’s Honestly Significant Difference Test was used to determine if differences from multiple comparisons were significant. One-way analysis of variance was used to compare mortality rates, pupation rates in the feeding experiments, and protein content in leaves. Origin 9.0 for Windows (Origin Lab Corp., Northampton, MA, USA) was used to create the graphs. The statistical package SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for all data analyses.

3. Results

3.1. Expression of Cry1Ah1 Toxin in Transgenic Poplar Leaves

Quantification of Cry1Ah1 protein using ELISA revealed that mature leaves from the six lines of transgenic poplar contained 4.1–10.6 µg/g of Cry1Ah1 protein. Protein content differed significantly among strains ($p < 0.05$). No Cry1Ah1 protein was detected in non-transgenic poplar leaves (control). The highest Cry1Ah1 protein content was found in the A-5-0 strain ($p < 0.05$) (Figure 4).
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**Figure 4.** Analysis of toxin protein contents in *Bacillus thuringiensis* (Bt) transgenic poplar. According to Tukey’s Honestly Significant Difference Test, the letters indicate statistical significance per ANOVA ($p < 0.05$) among different lines.

### 3.2. Effects of Bt-Cry1Ah1 Transgenic Poplar on Target Pests

One-day-old *H. cunea*, *M. troglodyta*, and *C. anachoreta* larvae were fed with Bt-Cry1Ah1 transgenic poplar or non-transgenic poplar leaves each day, and the corrected mortality rates were calculated (Tables 1–3). At the end of the experiments, the six transgenic lines exerted different insecticidal effects on the target insects. Among the six lines, A-5-0 exhibited the strongest insect resistance. The average corrected mortality associated with A-5-0 was 67.3% on day 18, 85.4% on day 12, and 87.0% on day 12 for *H. cunea*, *M. troglodyta*, and *C. anachoreta*, respectively. A-4-6 was also resistant to the three target insects (Figure 5A–C), with the corrected mortality of the target insects increasing with increased feeding time. Larvae of *H. cunea* that fed on transgenic poplar leaves experienced the most deaths on days 12 and 18, whereas larvae of *M. troglodyta* and *C. anachoreta* experienced the most deaths on days 6 and 12 (Figure 6). The pupation rates of the three target insects that fed on the six transgenic poplar lines differed significantly from that of the control ($p < 0.05$). The results indicate that transgenic poplar exhibited similar levels of resistance against *C. anachoreta* and *M. troglodyta*, which were higher than that against *H. cunea*.

#### Table 1. Insecticidal activity of *Bacillus thuringiensis* (Bt) transgenic poplar against *Hyphantria cunea*.

| Line   | Corrected Mortality (%) | Pupation Rate (%) |
|--------|-------------------------|-------------------|
|        | 6 d                     | 12 d              | 18 d              |
| CK     | 0.0 ± 0.0 a             | 2.2 ± 3.8 a       | 4.4 ± 3.8 a       | 91.1 ± 3.9 d |
| A-4-6  | 13.3 ± 6.7 a            | 27.3 ± 1.1 b      | 57.9 ± 8.4 cd     | 35.6 ± 3.9 a |
| A-5-0  | 17.8 ± 7.7 a            | 43.2 ± 3.3 c      | 67.3 ± 5.2 d      | 28.9 ± 3.9 a |
| A-3-4  | 11.1 ± 7.7 a            | 27.3 ± 6.8 b      | 46.5 ± 3.6 c      | 48.9 ± 3.9 b |
| A-5-23 | 11.1 ± 3.8 a            | 22.5 ± 9.8 b      | 48.7 ± 5.4 c      | 44.4 ± 3.9 b |
| X-2-0  | 4.4 ± 3.8 a             | 11.3 ± 7.6 b      | 25.5 ± 10.8 b     | 64.4 ± 10.1 c |
| Z-1-3  | 11.1 ± 7.7 a            | 22.4 ± 13.4 b     | 44.3 ± 5.1 c      | 48.9 ± 10.2 bc |

Values represent the means ± standard deviation (SD) of three independent experiments. According to Tukey’s Honestly Significant Difference Test, the letters indicate statistical significance per ANOVA ($p < 0.05$) among different lines in the same column.
Table 2. Insecticidal activity of Bt transgenic poplar against *Micromelalopha troglodyta*.

| Line     | Corrected Mortality (%) | Pupation Rate (%) |
|----------|-------------------------|-------------------|
|          | 6 d                     | 12 d              |
| CK       | 2.2 ± 3.8 a             | 8.9 ± 3.8 a       | 84.4 ± 10.2 d |
| A-4-6    | 22.5 ± 9.8 bc           | 68.3 ± 3.7 c      | 24.4 ± 3.9 ab |
| A-5-0    | 31.7 ± 7.2 c            | 85.4 ± 7.2 d      | 11.1 ± 7.7 a  |
| A-3-4    | 13.6 ± 0.5 b            | 63.4 ± 7.3 b      | 31.1 ± 3.8 b  |
| A-5-23   | 13.6 ± 6.7 b            | 58.5 ± 5.3 b      | 28.9 ± 3.9 b  |
| X-2-0    | 15.9 ± 10.1 b           | 51.3 ± 2.3 b      | 51.1 ± 10.2 c |
| Z-1-3    | 11.4 ± 4.1 b            | 38.9 ± 6.8 b      | 42.2 ± 7.8 bc |

Values represent the means ± standard deviation (SD) of three independent experiments. According to Tukey’s Honestly Significant Difference Test, the letters indicate statistical significance per ANOVA (p < 0.05) among different lines in the same column.

Table 3. Insecticidal activity of Bt transgenic poplar against *Clostera anachoreta*.

| Line     | Corrected Mortality (%) | Pupation Rate (%) |
|----------|-------------------------|-------------------|
|          | 6 d                     | 12 d              |
| CK       | 8.9 ± 3.8 a             | 15.6 ± 3.8 a      | 84.4 ± 3.8 d |
| A-4-6    | 16.8 ± 10.6 ab          | 73.9 ± 8.1 cd     | 20.0 ± 11.5 ab |
| A-5-0    | 24.4 ± 3.7 b            | 87.0 ± 8.7 d      | 11.1 ± 7.7 a  |
| A-3-4    | 16.8 ± 7.9 ab           | 63.0 ± 5.6 c      | 31.1 ± 3.8 b  |
| A-5-23   | 17.2 ± 5.1 ab           | 55.4 ± 2.6 c      | 37.8 ± 3.8 bc |
| X-2-0    | 9.7 ± 4.0 a             | 44.7 ± 8.1 b      | 46.7 ± 6.7 c  |
| Z-1-3    | 12.3 ± 4.5 a            | 47.2 ± 6.2 b      | 44.4 ± 3.8 c  |

Values represent the means ± standard deviation (SD) of three independent experiments. According to Tukey’s Honestly Significant Difference Test, the letters indicate statistical significance per ANOVA (p < 0.05) among different lines in the same column.

Figure 5. Effects of transgenic poplar on target insects. (A) Growth of *H. cunea* on days 8 (a) and 14 (b) of feeding on A-5-0 poplar, on days 8 (c) and 14 (d) of feeding on A-4-6 poplar, and on days 8 (e) and 14 (f) of feeding on control (CK) poplar. (B) Growth of *M. troglodyta* on day 10 of feeding on A-5-0 poplar (a), A-4-6 poplar (b), and CK poplar (c). (C) Growth of *C. anachoreta* on day 10 of feeding on A-5-0 poplar (a), A-4-6 poplar (b), and CK poplar (c). (D) Effects on *H. cunea* fed with control poplar for 2 (a), 4 (b), and 8 (c) days and with A-5-0 poplar for 2 (d), 4 (e), and 8 (f) days in the field. (E) Insect resistance exhibited by transgenic lines against *H. cunea* and *M. troglodyta* under field and laboratory conditions. According to Tukey’s Honestly Significant Difference Test, the letters indicate statistical significance per ANOVA (p < 0.05) among different lines.
Figure 6. Number of target insect deaths. Mortality of H. cunea larvae (A), M. troglodyta larvae (B), and C. anachoreta larvae (C) that fed on transgenic poplar for different durations.

One-day-old H. cunea and M. troglodyta larvae were fed Bt-Cry1Ah1 transgenic poplar or non-transgenic poplar leaves each day in the field, and corrected mortality rates were recorded. Mortality rates differed significantly between the treatments and the control ($p < 0.05$). Under field conditions, the strongest insect resistance was exhibited by A-5-0, with H. cunea experiencing 70.9% and 87.0% mortality on days 18 and 12, respectively. Based on field trials, A-5-0 had an obvious inhibitory effect on H. cunea feeding (Figure 5D). The corrected mortality rates of the larvae of the two species feeding on transgenic poplar in the field were higher than those recorded under laboratory conditions, but these differences were not significant (Figure 5E).

3.3. Effects of Bt-Cry1Ah1 Transgenic Poplar on a Non-Target Pest

Larvae of P. versicolora were fed with fresh transgenic poplar leaves in the laboratory to study its effects on this non-target insect (Figure 7A,B). On day 10, the corrected mortality rates of the fed larvae were 9.9–17.4%, with no significant differences in mortality observed between transgenic poplar and the control. The A-5-0 line, which exhibited a strong insecticidal effect on target insect species (H. cunea, M. troglodyte and C. anachoreta), had little effect on P. versicolora. The corrected mortality associated with A-5-0 was only 17.4%, not significantly different from that of the control. These results indicate that survival and development in P. versicolora are not affected when the larvae feed on transgenic poplar leaves.
Figure 7. Effects of transgenic poplar on a non-target insect. (A) Determination of insecticidal activity of transgenic poplar against *P. versicolora*. (B) Comparison of effects on *P. versicolora* on day 8 of feeding with CK poplar (a) and A-5-0 poplar (b).

### 3.4. Effects of Bt-Cry1Ah1 Transgenic Poplar on a Parasitoid Wasp

The emergence time of *C. cunea* in both the treatment and control was approximately 20 days after parasitism. There was no significant difference in emergence time between the treatment and the control (Figure 8A). Parasitoid wasps emerged at rates of up to 80%, with no significant difference in rate between the treatment and the control (Figure 8B). On average, more than 200 *C. cunea* wasps emerged in both the treatment and the control; no significant difference was recorded. The average weight of pupae fed on poplar was 100–150 mg. On average, the weight of pupae fed on non-transgenic poplar was higher than that of pupae fed on transgenic poplar (Figure 8C). The results indicate that the parasitic activities of *C. cunea* were not affected by toxin protein content in transgenic poplars. Thus, *Bt-Cry1Ah1* transgenic poplar does not have a significant effect on *C. cunea*.
Transgenic plants a protein. Among the six lines, transgenic poplar A-5-0 had the highest toxin protein content (µ poplar, which ranged from 0.12 to 21.00 proteins. Ding et al. (2018) determined the toxin protein contents in 17 lines of 2020 resistant to the insecticidal e toxic to lepidopterans. However, the potential risks to non-target insects should be assessed to ensure highly specific and are often only toxic to specific insect orders. In this study, Cry1Ah1 was only not appear to have significant e microorganisms through roots in the soil. At present, Bt H. cunea because di exhibited by each line might also have been related to the family of the target insects. No significant protein content. Among the six lines, transgenic poplar A-5-0 had the highest toxin protein content (>10 proteins) and exhibited the strongest insect resistance against target pests in both the field and laboratory.

In the feeding experiment, we found that the mortality of target insects increased significantly late into the feeding period, with the number of dead larvae increasing significantly at this stage. This might have been due to a large increase in food consumption at the later stage. As the larvae consumed more food, a larger amount of toxin protein was accumulated, leading to larval death. The insect resistance exhibited by each line might also have been related to the family of the target insects. No significant differences in corrected mortality were observed between the larvae of M. troglodyta and M. troglodyta (Lepidoptera, Lymantriidae) and H. cunea [29]. Our insect bioassays showed that the six transgenic poplar lines exhibited differing levels of insect resistance to three target insects. Significant differences among the different lines were related to different concentrations of toxin protein. Among the six lines, transgenic poplar A-5-0 had the highest toxin protein content (>10 µg/g) and exhibited the strongest insect resistance against target pests in both the field and laboratory.

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Bt transgenic plants can cause the death of target insects, but may also affect other organisms [31]. Transgenic plants affect non-target arthropods through leaves and stems above the soil, and soil microorganisms through roots in the soil. At present, Bt transgenic poplar, cotton, corn, and rice do not appear to have significant effects on non-target arthropods [32–34]. In general, Bt proteins are highly specific and are often only toxic to specific insect orders. In this study, Cry1Ah1 was only toxic to lepidopterans. However, the potential risks to non-target insects should be assessed to ensure

**Figure 8.** Effects of transgenic poplar on a parasitic natural enemy. (A) Emergence times of C. cunea. (B) Emergence rates of C. cunea. (C) Wasp output and pupa weight of the parasitoids.

### 4. Discussion

The ELISA analysis showed that the toxin protein content in transgenic poplar differed among lines, which was consistent with the results of the insect-resistance experiment. Differences in the expression of toxin proteins can be related to the transformation method and the materials and material components used [28]. The more insect-resistant lines expressed higher levels of toxin proteins. Ding et al. (2018) determined the toxin protein contents in 17 lines of Cry1Ac transgenic poplar, which ranged from 0.12 to 21.00 µg/g. The lines with higher toxin protein contents were more resistant to Lymantria dispar (Lepidoptera, Lymantriidae) and H. cunea [29]. Our insect bioassays showed that the six transgenic poplar lines exhibited differing levels of insect resistance to three target insects. Significant differences among the different lines were related to different concentrations of toxin protein. Among the six lines, transgenic poplar A-5-0 had the highest toxin protein content (>10 µg/g) and exhibited the strongest insect resistance against target pests in both the field and laboratory.
environmental safety. In China, *P. versicolora* is a major and widespread poplar leaf-eating pest. Thus, it is a good candidate for testing non-target effects of *Bt* transgenic poplar. In the laboratory experiment, *P. versicolora* did not exhibit negative effects after feeding on six lines of *Bt-Cry1Ah1* transgenic poplar. To date, *Bt* toxin has been shown to have limited toxicity against other insect groups. Nevertheless, additional multiple-generation feeding experiments and long-term field trials are needed. According to current findings, it is unlikely that *Bt* poplar is toxic to such non-target insect as *P. versicolora*.

The insect bioassays in which three target insects fed on transgenic lines clearly established that *Bt-Cry1Ah1* expression had a significant impact on target lepidopteran species. In general, significant effects on target insects from transgenic plants may consequently lead to direct or indirect effects on insect parasites such as parasitoid wasps. Transgenic insect-resistant plants mainly affect their natural enemies in two ways: (1) by delivering toxins when natural enemies feed directly on transgenic plant tissues such as pollen or leaves, and (2) indirectly affecting natural enemies by influencing their growth and development [37]. Few or no toxic effects of toxin proteins have been reported on woodlice, collembolans, mites, earthworms, nematodes, protozoa, and the activities of various soil enzymes. In studies in which *Harmonia axyridis* (Coleoptera, Coccinellidae) fed on *Chaitophorus populeti* (Hemiptera, Aphididae) that had consumed the leaves of *Cry3Ac + OC-1* transgenic *P. alba × P. glandulosa* in the laboratory [36], aphid consumption of transgenic plant materials did not have significant effects on the body mass, eclosion rate, mortality, sex ratio, or developmental time in the larval and pupal stages of the ladybird. Hu et al. (2007) reported that the species richness, abundance, and parasitism rate of the natural enemies of insect pests were higher in transgenic poplar forests compared to non-transgenic poplar forests. Moreover, the emergence rate did not differ significantly between the two groups. However, there are few reports about the effects of toxin proteins on parasitic natural enemies of insect pests [37]. There may be a cascade effect among species in which trophic interactions are strong between adjacent trophic levels, whereas interactions between connected, but non-adjacent, trophic levels are weakened due to the presence of intermediate trophic levels [38]. In our study, we found that there was no significant relationship between the parasitic behavior of the parasitoid and the species of plant leaves eaten by *H. cunea*. In maize, *Bt* transgenic and non-transgenic plants exerted similar effects on the parasitic behavior of *Trichogramma ostriniae* (Hymenoptera, Trichogrammatidae) [39]. In one study, however, a parasitic wasp was seemingly affected by a volatilized substance from the pupa [40]. By contrast, we found that pupae fed on transgenic poplar had no significant effects on parasitoid activity. Still, some differences were observed. Therefore, the toxin might have affected the pupae internally, producing volatilized substances. The transgenic poplar might not have transferred toxin proteins to the parasitic wasp through tertiary nutrition, or even if toxins were transferred, they might not have been toxic to *C. cunea*.

5. Conclusions

To our knowledge, this is the first report of transgenic poplar containing an insect-resistance gene incorporating codon optimization based on the optimal codon usage of poplar. The transgenic poplar lines investigated here exhibited resistance to three target insects and some resistance to non-target insects in both the field and laboratory. The results confirm that transgenic poplar exhibit good resistance to target pests and can be used for pest control in commercial poplar plantations.

Based on the results of our study, *Bt* transgenic poplar had significant insecticidal effect, which could effectively control the damage of Lepidoptera target insects, such as *H. cunea*, *M. troglodyta*, and *C. anachoreta*. The insecticidal effects of different transgenic poplar on target insects were different. Moreover, transgenic poplar had no obvious insecticidal effect on non-target insect of *P. versicolora*. There was no significant effect on parasitism of parasitoids *C. cunea*.

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