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Laboratory evaluation of effects of transgenic Bt corn pollen on two non-target herbivorous beetles, *Epilachna vigintioctopunctata* (Coccinellidae) and *Galerucella vittaticollis* (Chrysomelidae)

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Abstract

Laboratory bioassays were conducted to evaluate the effect of pollens from insecticidal transgenic Bt corn (event MON863) expressing Cry3Bb1 endotoxin on two native non-target herbivorous beetles, *Epilachna vigintioctopunctata* (Coccinellidae) and *Galerucella vittaticollis* (Chrysomelidae) larvae. Larvae were reared on leaf discs exposed to corn pollen for 10 days after hatching. *G. vittaticollis* had no adverse performance in terms of larval survival and development (proportion of larvae to third instar) between pollen doses of 500 and 2,000 grains/cm^2. Similarly, *E. vigintioctopunctata* did not show significant deleterious effects between pollen doses of 250 and 2,000 grains/cm^2. From the actual pollen doses deposited on leaf surfaces near cornfields, it was concluded that corn pollen from MON863 has no adverse effects on *E. vigintioctopunctata* and *G. vittaticollis* populations living on wild plants near cornfields.

Key words: Bt; corn pollen; Cry3Bb; non-target beetles

INTRODUCTION

Before the field cultivation of transgenic crops, the potential for ecological risk (e.g. hybridization with wild relative plants or increased invasiveness over crop fields) should be prudently evaluated based on available information and feasibility studies (Conner et al., 2003; Hancock, 2003). For insecticidal transgenic crops, especially transgenic corn expressing *Bacillus thuringiensis* (Bt) endotoxin, the effect of pollen exposure on non-target herbivorous insects should also be assessed (Lang, 2004).

With the short report by Losey et al. (1999) as a start, many researchers have studied the potential influence of pollen released from transgenic Bt corn on non-target lepidopteran, mainly the Monarch butterfly. These studies have revealed that only a small number of corn pollen are deposited onto the leaves of host plants and there are few risks to this butterfly in and near cornfields (Pleasants et al., 2001; Jesse and Obrycki, 2003; Anderson, 2004; Lang et al., 2004). The author also confirmed that the highest cumulative pollen dose on leaves was approximately 160 grains/cm^2 at 1 m from the edge of cornfields, and suggested the following procedure of risk assessment for Bt corn pollen: First, the Bt endotoxin level in corn pollen is evaluated by enzyme linked immunosorbent assay (ELISA), and then a bioassay using non-target insect larvae should be conducted in the laboratory when significant expression is detected in corn pollen (Shirai and Takahashi, 2005).

Following the development of Bt corn against lepidopteran pests, a new type of Bt corn, MON863, has been developed to target coleopteran pests, mainly the corn rootworm complex (*Diabrotica* spp. leaf beetles in North America) (Ward et al., 2005). In 2002, Monsanto Japan Limited submitted a petition for the import and cultivation of MON863 expressing Cry3Bb1 protein. The expression level of Bt toxin in MON863 corn pollen is relatively high, on average 77.1 μg/g (Duan et al., 2002) or 89.2 μg/g (Mattila et al., 2005) in fresh weight. At that time, a bioassay method using native non-target coleopteran species had not yet been developed in Japan, so the potential risk of pollen exposure to non-target beetles was evaluated

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based on an artificial diet overlay bioassay using larvae of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Yamane, 2005). Subsequently, Shirai and Takahashi (2005) developed a simple bioassay method to evaluate the effect of pollen exposure on non-target lepidopteran larvae, which is also suitable for risk assessment in coleopteran species.

The insecticidal spectrum of Cry3Bb1 is limited to small numbers of species among Coleopterans; purified Cry3Bb1 protein was toxic to larvae of *L. decemlineata* and *Diabrotica* spp. leaf beetles but not to larvae of weevils (*Anthonomus eugenii* Cano, *A. grandis* Boheman and *Callosobruchus maculatus* (Fabricius)) or *Tribolium castaneum* (Herbst) (Ward et al., 2005). In addition, a bioassay using purified Cry3Bb1 protein did not show adverse effects on two predatory ladybird beetles, *Hippodamia convergence* Guerin-Meneville and *Coleomegilla maculata* DeGeer (Ward et al., 2005). The potential risk from MON863 corn pollen was evaluated for *C. maculata*, which often uses corn pollen as a food source (Duan et al., 2002; Lundgren and Wiedenmann, 2002); however, other non-target herbivorous coleopterans which live on wild plants have not been evaluated. It will be worth evaluating the effect of pollen exposure on non-target herbivorous beetles near cornfields, because MON863 expresses a relatively high level of Bt toxin in pollen.

In this study, the effects of pollen exposure by Bt corn (MON863) on larval survival and development were evaluated in the laboratory for two native non-target herbivorous beetles, *Epilachna vigintioctopunctata* (Fabricius) (Coccinellidae) and *Galerucella vittaticollis* Baly (Chrysomelidae). These species were adopted because they are frequently found near cornfields in Japan, and are relatively easily reared in the laboratory.

MATERIALS AND METHODS

Collection of corn pollen. Transgenic corn, MON863AX, expressing Cry3Bb1 and isogenic non-transgenic corn, MON863AC, were planted in a segregated experimental field of the National Institute for Agro-Environmental Sciences (NIAES) in June, 2002. Before anthesis, all tassels were bagged and pollen was collected between July 31 and August 6. The pollen was sieved through a screen mesh to remove materials other than pollen and was then kept in a sealed plastic box at −30°C until the bioassay in fall 2003. For MON863AX, the presence of Cry3Bb endotoxin in pollen was confirmed with QuickStix Kit for YieldGard Rootworm Corn AS015LS (EnviroLogix, Portland, USA), but the expression level of the endotoxin in pollen was not quantitatively evaluated.

Insects and plant leaves. The herbivorous ladybird beetle, *E. vigintioctopunctata* was collected from an eggplant field in Itako City, Ibaraki Prefecture and was then successively reared using a potted black nightshade, *Solanum nigrum* (Solanaceae) in the NIAES laboratory by the rearing method of Shirai and Katakura (2000). *S. nigrum* plants for the bioassay were cultivated in an NIAES greenhouse. For the strawberry leaf beetle, *G. vittaticollis*, a population which was successively reared using bitter dock, *Rumex obtusifolius* (Polygonaceae) in the laboratory (Ohta et al., 1998), was used for larval bioassay. Another species of bitter dock, *R. japonicus*, grown in an NIAES field, was used as a food plant in this bioassay.

Preparation of corn pollen suspension. According to Shirai and Takahashi (2005), a corn pollen suspension containing the pre-selected pollen dose was prepared. Before larval bioassay, the number and weight of corn pollen grains on a micro-cover grass were determined with a stereomicroscope (E400, Nikon Eclipse, Tokyo, Japan) and a micro-balance (MX-5, Mettler Toledo, Tokyo, Japan) to weigh 1,000 grains of pollen, since pollen weight fluctuates under storage conditions. In this study, 20 μl of the pollen suspension was pipetted onto a leaf disc of 1.4 cm diameter (1.54 cm^2) for both *S. nigrum* and *R. japonicus* plants. Thus, 1,540 grains of pollen were required in a 20 μl suspension to create a leaf disc with a pollen dose of 1,000 grains/cm^2. When 1,000 grains of pollen weighed 0.245 mg, 18.86 mg ($=1.54\times0.245\times1{,}000\div20$) of pollen was added to 1 ml of distilled water in a vial. Unlike a leaf disc of wood sorrel, *Oxalis corniculata* (Shirai and Takahashi, 2005), pretreatment with a small amount of 80% acetone solution on the leaf disc was unnecessary since the leaf surfaces of *S. nigrum* and *R. japonicus* are hydrophilic.

Effect of corn pollen on larval survival of two herbivorous beetles. *E. vigintioctopunctata*:

Table 1. Larval survival and development of *Epilachna vigintioctopunctata* when exposed to transgenic Bt and non-transgenic corn pollens at different doses

Pollen dose (grains/cm^2)	Corn	No. replicate	Survival rate (%) at 10 days (Mean±SD)[a]	Proportion of larvae of the third instar (%) at 10 days (Mean±SD)[a]
0	leaf only	12	89.6±19.8 a	47.9±19.8 a
250	Bt	12	91.7±12.3 a	39.6±19.8 a
	Non-Bt	12	87.5±13.1 a	58.3±16.3 a
500	Bt	12	75.0±21.3 a	37.5±16.9 a
	Non-Bt	12	89.6±16.7 a	39.6±19.8 a
1,000	Bt	12	72.9±19.8 a	37.5±19.9 a
	Non-Bt	12	85.4±12.9 a	39.6±22.5 a
2,000	Bt	12	72.9±27.1 a	43.8±18.8 a
	Non-Bt	12	79.2±14.4 a	52.1±16.7 a

[a] Data were transformed to arcsin before statistical analysis. No comparison in the same column showed significant differences at the 5% level by Tukey's test.

Larval survival was evaluated at pollen doses of 250, 500, 1,000 and 2,000 grains/cm^2 of either transgenic Bt corn or non-transgenic corn, or with no pollen (only *S. nigrum* leaf), and 20 μl of pollen suspension containing the pre-selected dose was pipetted onto each leaf disc of *S. nigrum*. Each leaf disc was placed in a plastic sealed dish (9 cm diameter, 4.5 cm depth) with moist filter paper, and four first instar larvae (1 d after hatching) were introduced onto the leaf disc. The leaf discs were replaced daily, and larval survival and development were recorded for 10 days.

G. vittaticollis: Larval survival was evaluated at pollen doses of 500, 1,000 and 2,000 grains/cm^2 of either transgenic Bt corn or non-transgenic corn, or with no pollen (only *R. japonicus* leaf), and 20 μl of pollen suspension containing the pre-selected dose was pipetted onto each leaf disc of *R. japonicus*. Each leaf disc was placed in a plastic sealed dish (9 cm diameter, 4.5 cm depth) with moist filter paper, and five first instar larvae (1 d after hatching) were introduced onto the leaf disc. The leaf discs were replaced daily, and larval survival and development were recorded for 10 days.

All laboratory studies were conducted at NIAES under 25°C and 16L-8D conditions. Larval survival was not evaluated at a pollen dose of 3,000 grains/cm^2 for both beetles, since this pollen dose suspension was too dense to apply precisely onto a leaf disc using a micropipette.

Statistical analysis. Larval survival rates and the proportion of larval molting to the third instar at 10 days were transformed to arcsin and analyzed by Tukey's test using STATISTICA Version 5.5 (StatSoft Japan, Tokyo).

RESULTS

Larval survival and development of *E. vigintioctopunctata* and *G. vittaticollis*

At 250 grains/cm^2, larvae of *E. vigintioctopunctata* feeding on Bt corn pollen showed the highest survival rate (91.7%), not significant different from larvae feeding on non-Bt corn pollen or only *S. nigrum* leaf. Although survival rates on Bt corn pollen decreased slightly (72.9 to 75.0%) between 500 and 2,000 grains/cm^2, there were no significant differences in survival rates among non-Bt corn pollen doses. Larval development (proportion of larval molting to the third instar at 10 days) was not significantly different among comparisons (Table 1).

In *G. vittaticollis*, between 500 and 2,000 grains/cm^2, larvae feeding on either Bt corn or non-Bt corn pollen showed a high survival rate (more than 90.0%), not significantly different among comparisons. The proportion of larval molting to the third instar also showed no significant difference among comparisons (Table 2).

DISCUSSION

Insecticidal transgenic Bt corn targeted against lepidopteran pests, expressing high level endotoxin in pollen (e.g. Bt176), may have potential risks for non-target butterflies in and near cornfields, but

Table 2. Larval survival and development of *Galerucella vittaticollis* when exposed to transgenic Bt and non-transgenic corn pollens at different doses

Pollen dose (grains/cm^2)	Corn	No. replicate	Survival rate (%) at 10 days (Mean$\pm$SD)[a]	Proportion of larvae of the third instar (%) at 10 days (Mean$\pm$SD)[a]
0	leaf only	12	95.0±9.1 a	91.7±10.3 a
500	Bt	12	96.7±7.8 a	96.7±7.8 a
	Non-Bt	12	95.0±9.1 a	90.0±10.4 a
1,000	Bt	12	91.7±10.3 a	88.3±13.4 a
	Non-Bt	12	93.3±9.8 a	90.0±10.4 a
2,000	Bt	12	95.0±9.1 a	95.0±9.1 a
	Non-Bt	12	96.7±7.8 a	95.0±9.1 a

[a] Data were transformed to arcsin before statistical analysis. No comparison in the same column showed significant differences at the 5% level by Tukey's test.

MON810 or Bt11, expressing a low level in pollen (<0.09 μg/g being below the limit of detection by ELISA) did not possess any risk to non-target butterflies in and near cornfields (Kaplan, 2002; Mendelsohn et al., 2003). Bt corn (MON863) targeted against coleopteran pests expressed relatively high levels of the Cry3Bb1 endotoxin in corn pollen; average 77.1 μg/g (Duan et al., 2002) or 89.2 μg/g (63.1 μg/g after sieving) (Mattila et al., 2005) in fresh weight. Therefore, it is worth evaluating the effect of pollen exposure on non-target herbivorous beetles which live on wild host plants near cornfields.

This study investigated larval survival and development using leaf discs exposed to Bt corn pollen for 10-day periods since pollen deposition was most abundant at 3 to 7 d after anthesis and markedly decreased from 10 days in cornfields (Pleasants et al., 2001; Shirai and Takahashi, 2005). *G. vittaticollis* did not show any adverse larval survival and development between doses of 500 and 2,000 grains/cm^2 of Bt corn pollen. *E. vigintioctopunctata* showed a slightly decreased survival rate between doses of 500 and 2,000 grains/cm^2 of Bt corn pollen, but there was no statistical difference between larvae exposed to non-Bt corn pollen. The actual lethal dose such as LD_{50} was unable to be calculated in this study, since the larval bioassay was not conducted at more than 2,000 grains/cm^2. However, the highest cumulative pollen density on leaves throughout the flowering period was 150 grains/cm^2 on *S. nigrum* or 155 grains/cm^2 on sunflowers (*Helianthus annuus*) at 1 m from the edge of cornfields (Shirai and Takahashi, 2005). Other researches on the deposition of corn pollen also reported that the cumulative dose was 100 to 220 grains/cm^2 on milkweed (*Asclepias syriaca*) (Pleasants et al., 2001), or approximately 100 grains/cm^2 on wild carrot (*Daucus carota*) (Lang et al., 2004). It is unlikely that corn pollen of more than 2,000 grains/cm^2 is deposited on the leaf surface of *S. nigrum* or *R. japonicus* near cornfields.

Corn pollen expressing Cry3Bb1 endotoxin did not have any adverse effects on the life history parameters of the predatory and pollen-feeder ladybird beetle, *C. maculata* (Duan et al., 2002; Lundgren and Wiedenmann, 2002). The insecticidal targets of Cry3Bb1 endotoxin are limited to larvae of the Colorado potato beetle and some species belonging to the corn rootworm complex (*Diabrotica* spp.) (Ward et al., 2005), and adverse effects of this endotoxin on non-target coleopterans have not been documented. From this study, it was concluded that corn pollen from MON863 had no adverse effects on larvae of *E. vigintioctopunctata* and *G. vittaticollis*.

Following MON863, two Bt corn types targeted against coleopteran pests (DAS59122 expressing Cry34Ab/Cry35Ab and MIR604 expressing Cry3Aa) have been developed for commercial cultivation in the USA (USDA-APHIS, http://www.aphis.usda.gov/brs/not.reg.html). In the petition for the Japanese market, if these Bt corn types have a significant expression level of endotoxin in corn pollen, a potential risk assessment of pollen exposure is recommended using the procedure and native herbivorous insects used in this study. In addition to the risk assessment of corn pollen exposure in non-target herbivorous insects, more studies are needed to evaluate the potential risk for other non-target coleopteran species, especially useful predatory insects such as ground beetles (Carabidae), rove beetles (Staphylinidae) and ladybird beetles (Coccinellidae).

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