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Effects of a *Bacillus thuringiensis* toxin, two *Bacillus thuringiensis* biopesticide formulations, and a soybean trypsin inhibitor on honey bee (*Apis mellifera* L.) survival and food consumption

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Abstract – Newly emerged adult honey bees, *Apis mellifera* L., were fed with a pollen-based food containing various additives: purified and activated Cry1Ba δ-endotoxin, from *Bacillus thuringiensis* Bt4412 (Bt) (1, 0.25 and 0.025 % w/w), Bt biopesticide preparations, Dipel 2X (1 and 0.25 %) and Foray 48B (0.25 %), and Kunitz soybean trypsin inhibitor (SBTI) (1, 0.5 or 0.05 %). The bees received these foods for 7 days and were then given control food without additives for the rest of their lives. Bee survival time was unaffected, and the food was consumed at the same rate as control food for all treatments, except 1 % Dipel, where both survival and food consumption were significantly reduced. A second experiment showed that bees completely deprived of the pollen-based food also had poorer survival than those fed with the control food. Adult bees are unlikely to be harmed by transgenic plants expressing Cry1Ba or SBTI, or by Bt biopesticides that are used as recommended.

honey bee / *Bacillus thuringiensis* / Cry1Ba toxin / Kunitz soybean trypsin inhibitor / pest-resistant transgenic plants

1. INTRODUCTION

In response to concerns about the environmental and human health effects of chemical pesticides, as well as the evolution of pesticide-resistant insect biotypes, an increasing number of crop plants are being genetically modified to make them resistant to pest attack. Honey bees pollinate many of these crops, for example,

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clover (*Trifolium repens*), sunflower (*Helianthus annuus*), sweet potato (*Ipomoea batatas*), strawberry (*Fragaria* × *ananassa*), apple (*Malus domestica*), oilseed rape (*Brassica napus*), and cotton (*Gossypium* spp.) [11, 14]. The success of new transgenic cultivars will depend in part on their safety for pollinating insects.

Pollinating bees could be affected by pest-resistant transgenic plants either directly or indirectly. Direct effects may arise upon ingestion of pollen expressing or carrying the pest-resistance gene. Pollen is about 24 % protein [22] and thus represents a likely site for transgene expression. Cry1Ac toxin of *Bacillus thuringiensis* (Bt) has been expressed as 0.6 μg per gram of fresh weight (0.24 % of total protein) of transgenic cotton pollen [13]. However, neither serine nor cysteine proteinase inhibitors could be detected in the pollen of transgenic oilseed rape plants containing the genes for these inhibitors [20]. Nectar is unlikely to contain gene products as it is virtually pure carbohydrate and usually contains only a few amino acids [1]. There are no published records of gene products being found in the nectar of transgenic plants. Indirect effects may arise either via inadvertent changes in phenotype resulting from the position of the new gene in the plant genome (insertional mutagenesis) or via pleiotropic effects, whereby the expression of the new gene alters a biochemical pathway with phenotypic consequences. Pleiotropic effects would be expected to occur in every line of a transformed crop plant, whereas deleterious insertional mutagenesis effects may be avoided by selecting lines that do not have the undesirable change in phenotype. Reductions in nectar volume or concentration, or changes in flower morphology are examples of phenotypic changes which could indirectly affect bees [21].

White clover is an important forage crop and a significant nectar source for honey production [18]. Successful pollination of clover is also important in locations where clover dies during winter and adequate seed reservoirs in the soil must be maintained [26]. Two gene products are candidates for incorporation into transgenic white clover. The Bt δ-endotoxin Cry1Ba has been shown in laboratory experiments to be effective against the porina caterpillar, *Wiseana* spp. (Lepidoptera: Hepialidae) [23], and the proteinase inhibitor, SBTI (Kunitz soybean trypsin inhibitor), has been shown to be effective against the black field cricket, *Teleogryllus commodus* (Orthoptera: Gryllidae) [4], both of which are pests of white clover in New Zealand.

The present study examines the rate of pollen-food consumption and survival of newly emerged adult honey bees after feeding on two different pest-resistance gene products and two commercial Bt formulations. Pollen is a necessary protein source for newly emerged adult bees to complete their development, and of all bee life stages, these consume the greatest quantities of this food [31]. Thus, transgenic pollen is likely to have a greater impact on newly emerged adult bees than on larvae or older adults.

2. MATERIALS AND METHODS

Stocks of Italian race bees were obtained from our apiary at Mt Albert Research Centre, Auckland. Brood frames containing capped cells were brought into the laboratory and the cappings gently removed using forceps. Adult bees that were ready to emerge were gently pulled from their cells and held in groups of about 50 at 32 °C in darkness until enough had been collected for the experiment. This uncapping procedure was employed as a precaution against the bees becoming infected with *Nosema apis* Zander via by ingestion of spore-contaminated wax. All bees used in the experiments were less than 12 h old.

Two experiments were carried out. The first consisted of nine different treatments (three rates each of Cry1Ba, Bt biopesticides, or SBTI) and a control. The second consisted of one treatment (bees completely deprived of pollen-food) and a control. This experiment was undertaken to provide some information on the survival of bees starved of protein, with a view to assessing

whether effects observed in the first experiment could be explained as a simple avoidance of the pollen-food, rather than direct toxicity of the additives.

In the first experiment, bees were assigned randomly to wooden cages (9 × 8 × 7 cm) with mesh on two sides, 40 bees per cage. Thirty cages in total were set up: three blocks × nine treatments and one control. Each cage was fitted with two gravity feeders, one containing water and the other sugar syrup (60 % w/v sucrose solution), which were replenished as necessary during the experiment.

Sufficient pollen-food was prepared (0.33 parts pollen, 0.08 parts sodium caseinate, 0.16 parts brewer's yeast, and 0.43 parts sucrose mixed with water to a paste) to supply the total number of bees in each treatment group for about 8 days. The pollen used was bee-collected from unknown floral sources and stored at –20 °C.

For each cage, about 3 g of pollen-food with the appropriate treatment additive (described below) was placed in a plastic receptacle. This was weighed at the beginning of the experiment, at 12-h intervals for 5 days, daily for a further 4 days, and then every 2 or 3 days until all the bees had died. The numbers of surviving bees in each cage were recorded and dead bees removed at these times also. On day 7, each pollen-food receptacle was weighed, removed, and replaced with a new, weighed receptacle containing fresh pollen-food without any additive.

For the first experiment, Cry1Ba and SBTI (Sigma, St.-Louis, MO) were each mixed thoroughly into pollen-food at three concentrations. These were chosen to represent an unrealistically high concentration, a high but realistic concentration (equivalent to the highest expression level that might be expected to be effective in a transgenic plant with that gene) and a realistic low concentration (equivalent to a low, but still effective, plant expression level). For Cry1Ba, these were 1, 0.25 and 0.025 % w/w in pollen-food (equivalent to 4, 1 and 0.1 % of total protein), based on bioassay results with Bt-cotton and *Trichoplusia ni*, *Spodoptera exigua*, *Helicoverpa virescens*, and *Helicoverpa zea* [2, 19] and Bt expression levels in cotton pollen [13]. SBTI was used at 1, 0.5 and 0.05 % w/w in pollen-food (equivalent to 4, 2 and 0.2 % of total protein), based on bioassay results with SBTI-tobacco and *Spodoptera litura* (E.P.J. Burgess, unpublished data). Activated Cry1Ba toxin was obtained from a large-scale fermentation of *B. thuringiensis* Bt4412, purified and cleaved according to the method described by Simpson et al. [23]. Activated toxin was used as this most closely resembles the form in which Cry1Ba will be expressed in transgenic clover plants. Two biopesticides, Dipel 2X (Abbott, North Chicago, IL) and Foray 48B (Novo Nordisk, Danbury, CT), were added to pollen-food at 0.25 % w/w of active ingredient in pollen-food (equivalent to 1 % of total protein). This concentration was chosen as it approximates the minimum LD_{50} for a pesticide which is 'virtually non-toxic' to honey bees as defined by Crane and Walker [10], and it also allows comparison with the treatments where the gene products were delivered as 1 % of total protein. A further Dipel 2X treatment delivered an unrealistically high dose (1 % w/w of active ingredient or 4 % of total protein) to allow for direct comparison with the high-concentration gene product treatments.

A second experiment assessed the survival of bees starved of protein. It was set up in similar fashion, with eight cages in total: one control with pollen-food without additive and one protein-starvation treatment with no pollen-food × four blocks. Each cage had water and syrup provided, and was checked at regular intervals.

A survival curve, in which the percentage of bees remaining alive in each cage was plotted against time in days from the beginning of the experiment, was generated for each cage of bees. Mantel-Haenzel (log-rank) tests [15] were carried out to compare Kaplan Meier estimates of survival distribution, S(t), for bees receiving each treatment. Food consumption (mg per bee per 12 h) was calculated and, as the data had a skewed distribution, was transformed by log (value + 0.05). Mean transformed food consumption values for each treatment at each time point were compared by analysis of variance.

3. RESULTS AND DISCUSSION

In the first experiment only one treatment, Dipel at 1 % of active ingredient, resulted in significantly poorer bee survival than the other treatments (log-rank test, $P < 0.001$) (*figure 1b*). The bees in the second experiment that were starved of protein also had significantly poorer survival than their controls (log-rank test, $P < 0.001$) (*figure 1d*).

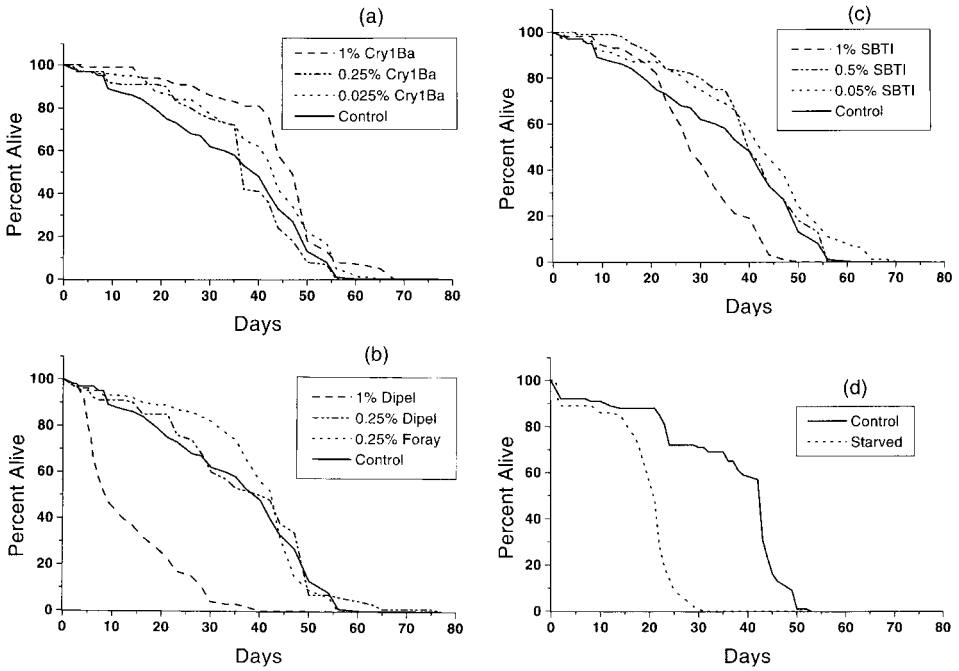

Figure 1. Survival of adult bees fed for 7 days with pollen-food containing a) 1, 0.25 or 0.025 % w/w Cry1Ba Bt toxin; b) 1 or 0.25 % Dipel or 0.25 % Foray (Bt biopesticides); or c) 1, 0.5 or 0.05 % SBTI proteinase inhibitor. Survival of control bees fed pollen-food with no additives is shown in each graph (a, b, c). The survival of bees deprived completely of pollen-food is compared with a second control group (d).

Food consumption rates were similar for all bees except those receiving 1 % Dipel (*figure 2b*). These bees consumed their food at a significantly lower rate than bees in the other treatments between days 3 and 6 (ANOVA, at day 3, morning, $P = 0.003$; at day 4, morning, $P < 0.001$; at day 5, morning, $P < 0.001$; and at day 6, $P < 0.001$). Later in the experiment, when all bees were receiving control food, these bees consumed significantly more than the others (ANOVA, at day 21, $P = 0.022$; at day 26, $P = 0.002$; at day 28, $P = 0.009$; at day 30, $P = 0.035$; and at day 33, $P = 0.004$).

Thus, bees were not harmed by ingesting the gene products tested here, even at concentrations much higher than the expected expression levels in transgenic plants. Neither were bees harmed by realistic concentrations of two commercial Bt formulations. The mortality and reduced food consumption observed among bees receiving an unrealistically high concentration of Dipel 2X serves to demonstrate that the methods used in these experiments were appropriate for demonstrating toxic effects of the additives.

The safety of commercial Bt formulations for honey bees was previously established [3, 7–9, 12, 17, 27]. Only preparations containing exotoxin, in addition to the endotoxins encoded by *cry* genes, have been shown to have any harmful effects on bees [16, 28]. Additionally, Bt is used for control of a lepidopteran pest of bee hives, the greater wax moth, *Galleria mellonella* [6,

25, 29, 30], further suggesting a lack of susceptibility of honey bees to Bt.

In the present study, only 1 % Dipel (which does not contain any exotoxins) resulted in reduced food consumption and significant bee mortality. Although a statistical comparison cannot be made between results from the two different experiments, it is interesting to note that the median longevity of bees fed 1 % Dipel (12 days with 95 % confidence interval of 8–16 days) was lower than that of bees that were starved of protein (21 days with 95 % confidence interval of 21–22 days). This suggests that their mortality was not simply a result of the bees being repelled by the Dipel in the diet, but that it also had some toxic effect. However, we did not ascertain whether this effect was due to the Bt spores and toxins in the preparation or to one of the 'inert' ingredients in the mixture.

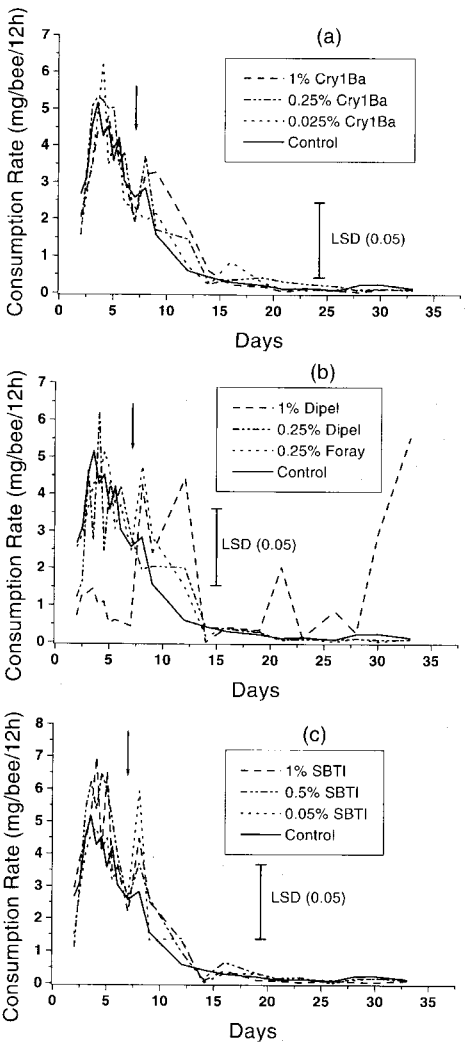

Figure 2. Rates (mg/bee/12 h) at which adult bees consumed pollen-food containing a) 1, 0.25 or 0.025 % w/w Cry 1Ba Bt toxin; b) 1 or 0.25 % Dipel or 0.25 % Foray (Bt biopesticides); or c) 1, 0.5 or 0.05 % SBTI proteinase inhibitor. The rate of consumption by control bees of pollen-food without additives is shown in each graph (a, b, c). After 7 days bees in all treatments were transferred to control food (arrows).

One important difference between Bt-transgenic plants and Bt biopesticides is that the transgenic plant will express only a single Bt toxin, or a well-defined combination of toxins, whereas a biopesticide may contain several toxins in unknown proportions, as well as spores and vegetative stages. Furthermore, Bt-transgenic plants will express only the soluble and cleaved form of the toxin, rather than the full-length and crystalline forms found in commercial Bt preparations. There are few published studies describing honey bee tests with purified Bt toxins that represent single *cry* gene products. Our results agree with those of Sims [24], who used full-length purified Cry1Ac toxin and found that the mortality (24 %) of adult bees fed 20 $\mu g \cdot mL^{-1}$ of this toxin in syrup for 7 days did not differ significantly from that of control bees fed either heat-attenuated toxin (22 %) or no toxin (25 %). These mortality figures are higher than those recorded in our study after 7 days. This may have been because the bees in Sims' study were kept at low temperatures (22–26 °C) and were not supplied with any pollen-based or other protein food.

Consumption of syrup was not recorded here or in previously published studies, but if we assume that caged bees consume 0.032 mL of syrup per bee per day (L.A.M.,

unpublished data), then the bees in Sims' study received 0.64 μg of full-length Cry1Ac protein per day. This represents a lower dose of Cry protein than any received by bees in our own study. Averaged over their lifetimes, our bees received 0.95, 11 or 40 μg of cleaved Cry1Ba per day (equivalent to 0.1, 1 or 4 % of total protein, respectively). Cry1Ba expression levels that will result in effective pest control on transgenic white clover have not yet been established. However, Bt-cotton plants expressing Cry1Ab or Cry1Ac as 0.05–0.1 % of total soluble plant protein have been shown to effectively control the pest insects, *T. ni* and *S. exigua* [19]. If these levels are typical of Bt-transgenic plants, then we can assume that pest-resistant white clover expressing Cry1Ba will not harm adult bees.

The SBTI results recorded here suggest that this gene product will also be safe for adult honey bees, as none of the treatments reduced longevity or food consumption significantly. This is in agreement with an earlier study [5] in which adult bees were supplied ad libitum with sugar syrup to which SBTI had been added at various concentrations. To compare results from the two studies, the lifetime doses of SBTI consumed in each may be estimated. In the present study, this can be determined by multiplying the mean amount of pollen food consumed over the first 7 days of the experiment by the concentration of SBTI in the food. This gives three treatments delivering total doses of 866, 451 and 36 μg of SBTI (1, 0.5 and 0.05 % treatments, respectively). None of these treatments caused significant bee mortality. In the earlier study [5], assuming that bees consume 0.032 mL of syrup per day, the lifetime doses of SBTI can be estimated by multiplying the median lifetimes of the bees in each treatment by 0.032 ($mL \cdot day^{-1}$) and by the concentration of SBTI in the syrup ($mg \cdot mL^{-1}$). This gives three high doses, 1.92, 2.24 and 0.77 mg, which caused significant bee mortality and two low doses, 122 and 12 μg, which did not. Thus, it appears that SBTI will be safe for adult bees provided the lifetime dose received by each bee is less than a 'threshold' dose somewhere between 700 and 900 μg of SBTI. Given that nectar would be unlikely to contain SBTI, pollen of a transgenic plant would have to be expressing SBTI as 4 % of total protein or more to have the possibility of adversely affecting bee longevity. It seems unlikely that transgenic SBTI-plants will express SBTI at 4 % or more of total protein because transgenic SBTI-tobacco plants have been shown to reduce the growth of the pest *S. litura* when expressing SBTI at 0.2–0.4 % of total protein and to kill this insect at 0.4–1 % (E.P.J. Burgess, unpublished data).

In conclusion, it is unlikely that transgenic white clover expressing either Cry1Ba or SBTI for protection against pest attack will be toxic to adult honey bees. However, further investigation is required to establish whether the same lack of toxicity would be observed in bees kept under field conditions. Furthermore, sub-lethal effects, particularly on foraging behaviour, were not studied here but could have significant effects on colony survival. Inadvertent changes in clover phenotype that may indirectly affect bees, such as changes in flower morphology, nectar volume or nectar concentration, may be avoided by careful testing and selection of the transgenic lines prior to field release.

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Résumé – Effets d'une toxine de *Bacillus thuringiensis*, de deux formulations de biopesticide à base de *Bacillus thuringiensis* et d'un inhibiteur de trypsine soja sur la survie et la prise alimentaire de l'abeille mellifère (*Apis mellifera* L.). Des abeilles fraîchement écloses ont été encagées (40/cage) et ont reçu un sirop de sucre (60 % masse/volume) et de l'eau ad libitum et une nourriture à base de pollen et de divers additifs. Les additifs utilisés étaient les suivants : la δ-endotoxine Cry1Ba purifiée et activée, venant de *Bacillus thuringiensis* Bt4412 (Bt) (1, 0,25 et 0,025 % masse/masse), deux préparations biopesticides de Bt : Dipel 2X (1 et 0,25 %) et Foray 48B (0,25 %) et l'inhibiteur de trypsine soja de Kunitz (SBTI) (1, 0,5 ou 0,05 %). Les concentrations de Cry1Ba et de SBTI ont été choisies de façon à correspondre à trois niveaux d'expression dans les plantes transgéniques : un niveau élevé non réaliste et deux concentrations que l'on peut s'attendre à trouver dans les plantes exprimant ces protéines à des niveaux efficaces contre les insectes déprédateurs. Les concentrations en Cry1Ba équivalent à des niveaux d'expression dans la plante de 4, 1, et 0,1 % de la protéine totale et celles de SBTI à 4, 2 et 0,2 % de la protéine totale. Le traitement au Dipel à 1 % équivaut à 4 % de la protéine totale et se situe bien au-dessus des niveaux de pulvérisation recommandés pour ce biopesticide. Les traitements au Dipel et au Foray à 0,25 % équivalent à 1 % de la protéine totale et s'approchent de la DL_{50} pour un pesticide qui est « virtuellement non toxique » pour les abeilles. Trois blocs de neuf traitements et un témoin ont été mis en place (30 cages au total). Les abeilles ont été maintenues en étuve à 32 °C. Elles ont reçu les aliments durant sept jours, puis la nourriture témoin sans additifs pendant le reste de leur vie. La mortalité des abeilles dans les cages a été vérifiée et les récipients de nourriture à base de pollen ont été pesés toutes les 12 h durant cinq jours, puis chaque jour durant les quatre jours suivant et trois fois par semaine jusqu'à ce que les abeilles meurent. La survie des abeilles n'a pas été affectée par les traitements et la nourriture avec additif a été consommée au même rythme que la nourriture témoin pour tous les traitements, sauf celui au Dipel à 1 % (*figures 1b* et *2b*). Une seconde expérience a montré que les abeilles totalement privées de nourriture à base de pollen survivaient moins longtemps que celles ayant reçu la nourriture témoin (*figure 1d*). Nous concluons qu'il est peu probable que les plantes transgéniques exprimant Cry1Ba, SBTI ou les biopesticides utilisés aux doses recommandées présentent une toxicité directe pour les abeilles adultes. Néanmoins il serait souhaitable de procéder à d'autres études sur des abeilles en conditions naturelles et d'examiner les effets sub-létaux.

***Apis mellifera* / *Bacillus thuringiensis* / toxine Cry1Ba / inhibiteur de trypsine soja Kunitz / plante transgénique / toxicité**

Zusammenfassung – Wirkung eines Gifts von *Bacillus thuringiensis*, Wirkung zweier Formulierungen von Biopestiziden auf der Basis von *B. thuringiensis* sowie eines Trypsinhemmers aus Sojabohnen. Frisch geschlüpfte Honigbienen (*Apis mellifera* L.) wurden in kleine Käfige überführt (40 Bienen pro Käfig), die Zuckerwasser (60 % m/V) und Wasser ad libitum enthielten. Zusätzlich wurde ein auf Pollen basierendes Futter mit unterschiedlichen Zusätzen geboten. Als Zusätze wurden gegeben: a) gereinigtes und aktiviertes Cry1Ba δ-Endotoxin vom *Bacillus thuringiensis* Bt4412 (Bt) (1, 0,25 und 0,025 % w.w), b) Präparate von Bt Biopestiziden, Dipel 2X (1 und 0,25 %) und Foray 48B (0,25 %), und c) Kunitz Sojabohnen Trypsin Hemmer (SBTI) (1, 0,5 oder 0,05 %). Die Konzentrationen von Cry1Ba und SBTI wurden entsprechend 3 Stärken der Expression in transgenen Pflanzen gewählt: ein unrealistisch hohes Niveau und 2 Konzentrationen, wie sie in Pflanzen erwartet werden

könnten, wenn sie gegen Insektenbefall wirksam sein sollen. Die Cry1Ba Konzentrationen entsprechen den Expressionsniveaus von Pflanzen mit 4, 1 und 0.1 %, die von SBTI entsprechen 4, 2 und 0,2 % des Gesamtproteins. Die 1 % Dipel Behandlung entspricht 4 % des Gesamtproteins und liegt weit über der empfohlenen Sprühmenge für dieses Biopestizid. Die 0,25 % Dipel und Foray Behandlungen entsprechen 1 % des Gesamtproteins und liegen nahe dem Minimum der LD_{50} von Pestiziden die für Honigbienen als praktisch ungiftig gelten. Drei Blöcke dieser 9 Behandlungen und eine Kontrolle wurden durchgeführt (insgesamt 30 Käfige). Die Bienen wurden im Brutschrank bei 32 °C gehalten. Sie wurden 7 Tage lang mit den Zusätzen gefüttert, danach erhielten sie für den Rest ihres Lebens Kontrollfutter ohne Zusatz. In den Käfigen wurde der Totenfall kontrolliert. Die Behälter mit dem Pollenfutter wurden 5 Tage lang in 12stündigem Abstand, weitere 4 Tage täglich und danach 3 mal pro Woche gewogen, bis alle Bienen tot waren. Die Überlebensdauer der Bienen blieb unbeeinflusst, und das Futter wurde bei allen Behandlungen in gleichen Mengen wie bei den Kontrollen aufgenommen, mit Ausnahme von 1 % Dipel, bei dem beides, Überlebensrate und Futterverbrauch, signifikant reduziert waren (*Abbildung 1b* und *2b*). Ein 2. Versuch zeigte, dass Bienen ohne Pollenfutter schlechter überlebten als die mit Kontrollfutter (*Abbildung 1d*). Wir schließen daraus, dass transgene Pflanzen, die Cry1Ba oder SBTI erzeugen, oder auch die Bt Biopestizide, die nach Vorschrift angewendet werden, wahrscheinlich keine direkte giftige Wirkung auf adulte Bienen haben. Trotzdem wären weitere Studien mit Bienen unter Feldbedingungen, unter Einschluss von Prüfungen von sublethalen Wirkungen, wünschenswert.

Honigbienen / *Bacillus thuringiensis* / Cry1Ba Gift / Kunitz Sojabohnen Trypsinhemmer / krankheitsresistente transgene Pflanzen

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