Assessment of effects of Bt-oilseed rape on large white butterfly (*Pieris brassicae*) in natural habitats

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Abstract

Much effort has been allocated to the definition of risk, relevant for the assessment of genetically modified plants. However, few studies have emphasised the limitations in testing methods. In this study, tests for and effects on non-target herbivores were exemplified and evaluated for *Pieris brassicae* (L.) (Lepidoptera: Pieridae) and a genetically modified *Brassica napus* L. (Brassicaceae) expressing the *Bacillus thuringiensis* (Bt)-toxin Cry1Ac. It was established that this herbivore recognises and accepts the transgenic plant as a host. It was found that ovipositing females of *P. brassicae* preferred the transgenic variety for egg-laying. Therefore, effects of the transgenic host plant on the herbivore were determined. Larvae feeding on the Bt-plants experienced 100% mortality for all larval stages. Based on these observations, a population model was established. The model showed that larval survival is increased with amount of food (number of plants) and reduced with the frequency of transgenic specimens, number of host plants needed for completing larval development, and number of egg-laying butterflies. Such models may both aid the design of further tests for effects and support the assessment whether population effects are likely to occur due to the presence of insect-resistant plants outside the agricultural area.

Introduction

Genetically modified insect-resistant plants are designed to be effective against herbivorous insects feeding on the crop, and effects on the target organisms within the agricultural fields are intended. However, if the insect-resistant plant spreads into natural habitats, unwanted ecological effects of such an invasion may arise if the transgenic plant has detrimental effects on insects that feed on it. Therefore, it is necessary to estimate the magnitude of the possible effects on the naturally occurring herbivorous insects.

In this study, the large white cabbage butterfly, *Pieris brassicae* (L.) (Lepidoptera: Pieridae), and one of its host plants of the Brassicaceae family are examined as a model system for estimation of the effects on herbivorous insects. *Pieris brassicae* is present both in agricultural and non-agricultural habitats (Shreeve, 1981), where the larvae feed on plants of the Brassicaceae (Feltwell, 1982). The butterfly lays large egg clutches, so that normally the host plant becomes totally defoliated and the larvae are forced to migrate to other host plants to complete larval development (Davies & Gilbert, 1985). Consequently, they have a comparatively high risk of encountering a transgenic plant during their development. The transgenic crop used in this study was an insect-resistant Bt-oilseed rape, *Brassica napus* L. Oilseed rape is known to form feral populations in natural and semi-natural habitats, given a certain amount of disturbance (Crawley & Brown, 1995; Pessel et al., 2001). Furthermore, competition experiments have shown that Bt-oilseed rape may out-compete insect-susceptible plants at high herbivore densities (Damgaard & Kjær, 2009).

It is commonly agreed that a scientific risk assessment should form the basis for a rational decision for each genetically modified plant (GMP), i.e., a case-by-case assessment. It is also generally accepted that the most efficient way to do this is to apply a tiered approach, meaning
that, initially, relatively simple assessments are carried out to evaluate whether effects may occur. If this simple approach suggests that a certain risk may occur, then a more demanding and realistic testing procedure is conducted at the next tier to determine risk probability (Andow, 1990; Poppy, 2000; Andow & Hilbeck, 2004).

In the following, we have employed a stepwise approach to assess effects on non-target herbivores. The first step establishes whether the herbivore recognises and accepts the transgenic plant as a host. Dependent of the outcome, effects of the transgenic host plant on the herbivore are assessed (lethal or sub-lethal). Finally, if the herbivore accepts the transgenic plant as a host and the plant has adverse effects, it is evaluated to which extent the transgenic crop affects the population size of the herbivore.

**Materials and methods**

**Plants and insects**

Three varieties of canola were used in the experiments. The test plants were: B. napus var. Westar, B. napus var. Iris, and W45, a genetically modified B. napus var. Westar, expressing the Bacillus thuringiensis (Bt)-toxin Cry1Ac (Stewart et al., 1996). A laboratory stock of the large white cabbage butterfly, P. brassicae, was reared on B. napus var. Iris. Adult butterflies were kept in large cages (70 × 100 × 100 cm) in a greenhouse. The butterflies were supplied with 10% (wt/vol) sucrose water in artificial flowers as well as oilseed rape plants for oviposition. Seeds of B. napus were germinated in Petri dishes, and the seedlings were transplanted to 9-cm pots and placed in a greenhouse. The plants were grown with 12-h photoperiods at an average temperature of 20 °C (range: 14–37 °C) and 64% r.h. (48–83%). The soil used was a completely fertilised compost mixture (SM-grøn; Stenrøgel Mosebrug, Kjellerup, Denmark). The plants were watered from the bottom with fertilised water (14:3:25 NPK, 100 g l⁻¹; ion, 10 ml l⁻¹; phosphoric acid, 25 ml l⁻¹).

**Protein expression in the W45 plants**

Prior to experimentation, the content of Cry1Ac in the leaves of the W45 plants was determined by means of enzyme-linked immunosorbent assays on leaf material of varying age. Five samples were collected on each of five sampling dates. The content ranged between 0.029 and 0.6% with an average content of 0.2%. Figure 1 shows that the content is reduced as the plant grows older.

**Oviposition experiments**

A cage experiment was conducted to assess whether the females showed preference to plants containing Bt-toxins or a conventional oilseed rape variety. The cages (70 × 100 × 100 cm) were placed in a greenhouse, at L16 (22 °C):D8(18 °C) h. Three specimens of each plant type, i.e., Iris and W45, were placed within each cage and, subsequently, three males and four females were released into the cage for oviposition. The cage also contained artificial flowers with 10% sucrose water as food for the adult butterflies. Egg clutches produced were counted for each plant type over a 7-day period.

Data were analysed using a one-sample χ²-test (Siegel & Castellan, 1988).

**Larval host preference**

Experiments were conducted to assess the acceptance/deterrence of Bt-plants and conventional oilseed rape varieties. All tests were performed with six leaf discs (3.5 cm diameter) placed in the perimeter of a glass Petri dish (14 cm diameter) lined with humid filter paper. Five third instars were weighed and added to the centre of each dish. Larvae had access to the leaf discs for 1 h. The test consisted of three set ups: (1) no-choice test with each of the three plant varieties (six discs of the test plant); (2) combinations of the three varieties presented for the larvae in pairs (three discs of each test plant); and (3) all three varieties in a multiple choice arena (two discs of each test plant). Each test was replicated 25 times. The area of the leaf discs was measured immediately after the test (LI 3100 area meter; LI-COR Biosciences, Lincoln, NE, USA) and the consumed leaf area was estimated.

Consumption per Petri dish and relative intake per food type in the single treatment were found to be normally distributed. Initially, overall treatment effects on consumption (differences in total leaf area consumed per test arena) and larval weight were compared by means of two-way ANOVA. However, because there was no significant effect...
of larval size and no interaction between larval weight and treatment, larval weight was omitted from the analysis. Therefore, one-way analyses were conducted and differences between treatments were compared by means of Tukey’s honestly significant difference (HSD) t-test.

**Feeding experiments to assess stage-specific toxicity of Bt-plants**

Because larvae of the large white cabbage butterfly migrate to other host plants during larval development, they may encounter a transgenic plant in different phases of their development. Therefore, feeding experiments were conducted to assess the toxicity of W45 to first, third, and fifth instars. Toxicity was measured as relative survival. For each instar, five groups of 10 homogeneously sized larvae were assigned to W45 plants. Prior to the experiment, many larvae were fed control food (whole leaves of Westar). After reaching a specific instar, they were placed on plants of the transgenic variety. Food plants equal in age were used to obtain similar content of the Cry1Ac for all test animals. Generalised linear models for repeated measures were used to test whether different larval instars had different survival curves when feeding on W45. The link function model for this was logit, as mortality data are binomially distributed.

**Modelling herbivore survival**

The experimental results in this study indicated that: (1) ovipositing females accepted both transgenic and non-transgenic plants for egg-laying, (2) larvae did not discriminate against transgenic plants, and (3) transgenic plant material was highly toxic to the larvae in all instars. Using these results, it was calculated under which circumstances the herbivore was likely to experience a significant decline in population size. Because the herbivore displayed a simple behaviour in that both ovipositing females and larvae accepted Bt-plants as well as conventional varieties as hosts, and none of the instars survived on Bt-plants, we chose a simple probabilistic modelling approach. The host plants were separated into poisonous and non-poisonous specimens, and if a larva ate from a poisonous plant, it was assumed to die. In many ecological settings, a larva has to feed on more plants than the plant it hatched on (Davies & Gilbert, 1985). The number of plants a larva has to visit, in addition to the plant on which it hatched, is denoted as $b$. This parameter is a complicated function of number of eggs, size of the host plant, and several other biotic and abiotic factors that determine survival and/or developmental rate of plants and herbivores, such as, e.g., presence of predators and parasites or various climatic regimes.

A host population is divided into several sub-populations. The area of such a host sub-population is defined by the maximum distance larvae may disperse. If a larva hatches in a given sub-population, it is not expected to leave the sub-population before it becomes a mature butterfly. The area that the sub-population covers does not affect the result of the present simulation, as the sub-population is defined by a number of host plants and not by the area.

Each sub-population consists of $n$ host plants, of which some are poisonous with a probability $q$. Each sub-population is visited by $m$ butterflies, which each lay $k$ eggs on a random host plant. A butterfly is assumed not to lay eggs on host plants where eggs are already present, because the large white cabbage butterfly possesses oviposition deterring pheromones (Schoonhoven et al., 1981) and/or plants with eggs produce oviposition deterring compounds (Blaakmeer et al., 1994).

The probability that an egg develops to adulthood is the probability that the egg is laid on a non-poisonous plant $(= 1 - q) \times$ the probability of sampling $b$ non-poisonous plants out of $n$ plants without replacement (the hyper geometric distribution). Thus, the probability that an egg survives to adulthood is:

$$P = (1 - q) \left( \frac{(n-m)(1-q)}{b} \right) / \left( n-m \left( 1 - q \right) \right), \quad (1)$$

where $(n-m)(1-q)$ is the number of non-toxic plants available for feeding, and $n - m(1-q)$ is the number of plants available, including the toxic ones, noting that $m(1-q)$ non-poisonous plants are eaten before the first larval migration to new plants.

**Spatial aggregation of host plants**

In the above hyper geometric distribution, it was assumed that the number of plants, $n$, was constant among sub-populations. However, normally, plants are not evenly distributed in natural habitats, but more or less patchily. To evaluate the importance of spatial aggregation of plants, the above probabilistic model was simulated with the number of host plants varying; it was assumed to be distributed either randomly (Poison) or in patches (negatively binomial). The proportion of poisonous plants in each sub-population was assumed to be binomially distributed with the same mean frequency of poisonous plants for each sub-population.

**Non-random distribution of poisonous host plants**

A second form of spatial aggregation may occur if the frequency of poisonous plants is allowed to vary among sub-populations. To study the effect of such a non-random distribution of poisonous plants on herbivore survival, $n$ was fixed, while $q$ was allowed to vary between sub-populations. The frequency of poisonous plants ($q$) was described by a beta-distribution with two parameters ($\alpha$, $\beta$).
β). While β was kept fixed, α varied, to obtain a constant mean value of q. Two levels of variation were created, afterwards referred to as ‘variable q’ (β = 10) and ‘highly variable q’ (β = 4). The simulation models were implemented in Mathematica (Wolfram, 1996), and for each parameter combination, the average number of surviving larvae from 100 runs with 100 sub-populations was recorded.

Results

Oviposition

The ovipositing females were not repelled by the transgenic variety (W45) for egg-laying, in fact, it was the preferred host. Out of the 60 egg clutches produced during the experiment, 41 were found on W45 (χ² = 8.08, d.f. = 1, P<0.01).

Larval host preference

Total consumption was similar between treatments, except for the no-choice trial with W45 leaf discs. In this treatment, food intake was reduced with approximately 50% compared to all other treatments (Table 1). In the dual- and triple-choice trials, it was observed that W45 was not eaten to the same extent as the other food types. In all trials including W45 as a choice, measurable consumption occurred on W45, despite the fact that significantly more was eaten from the other plant varieties.

Feeding experiments to assess stage-specific toxicity of Bt-plant

Feeding on W45 resulted in 100% mortality for all tested instars. The three larval stages responded differently (ANOVA: F11,2 = 120.4, P<0.0001). The younger the larvae, the more susceptible they were to the toxin (Figure 2).

Modelling population effects

Generally, survival probability of larvae increases with number of host plants (n) and decreases with frequency of transgenic plants (q), number of new plants needed for full larval development (b), and number of visits of ovipositing butterflies (m). However, survival probability is mainly determined by q and b (Figure 3), and n and m only have a minor effect, as long as the number of plants, n, is much higher than b, and the number of ovipositing butterflies, m, is much lower than n (results not shown).

It was found that when a sub-population consisted of five plants on average, then the insect population responded to random and patchy plant distributions in a

| Table 1 | Feeding of third instars of *Pieris brassicae* in 1-h choice experiments (no-choice, dual choice, and triple choice) on *Brassica napus*. Iris and Westar are conventional *B. napus* varieties and W45 is a transgenic variety that expresses the Bt-toxin Cry1Ac |
| --- | --- | --- |
| Food type | Average intake per Petri dish (cm²) n | Average intake per food type (cm²) d.f. F P |
| No-choice experiment | | |
| Iris | 8.40a | 25 |
| Westar | 7.97a | 25 |
| W45 | 3.12b | 25 |
| Dual-choice experiment | | |
| Iris-W45 | 7.23a | 25 | 1 | 6.63 | 0.017 |
| Iris | 7.11a | 4.71a | 2.52b |
| W45 | 7.62a | 25 | 1 | 32.45 | 0.0001 |
| Westar-W45 | 5.57a | 2.05b |
| Westar | 4.14a |
| W45 | 1.07b |
| Iris-Westar | 7.71a | 25 | 1 | 0.68 | 0.42 |
| Iris | 3.57a |
| Westar | 4.14a |
| Triple-choice experiment | | |
| Iris-Westar-W45 | 8.45a | 25 | 2 | 35.16 | 0.0001 |
| Iris | 4.03a |
| Westar | 3.35a |
| W45 | 1.07b |

Differences in consumption between varieties were tested by means of one-sample t-test. The choice experiments were tested by means of one-way ANOVA, followed by Tukey’s HSD test for differences between treatments. Different letters within an experiment following intake values indicate a significant difference between varieties.
similar fashion (Figure 4) and fewer larvae survived when compared to a fixed number of plants per sub-population. For higher n, i.e., 10 and 15, only negligible differences were observed between random, patchy, and fixed distribution of host plants (Figure 4). Simulations with higher b did not change this pattern (data not shown).

**Non-random distribution of poisonous plants**

Again, no major differences were observed between the tested plant distributions at low plant densities (Figure 4). At densities of 10 or 15 plants per sub-population, a consistent, but small, increase in the insect population was observed in plant populations where the two plant types were unevenly distributed, compared to a fixed density with homogenous distribution of the two plant types.

**Discussion**

We found that it is important to assess the exposure condition for test organisms in terms of ovipositional behaviour of female and larval feeding behaviour. Population assessments can aid the design of tier-2 assessments.
Host acceptance

Ovipositing females use both Bt-plants and the conventional variety as host plants. Liu et al. (2002), Bernal & Setamou (2003), and Van den Berg & Van Wyk (2007) all found that the same holds true for the response of pink bollworm to Bt-cotton, the response of the stem borers Eoreuma loftini Dyar and Diatraea saccharalis (Fabricius) to sugarcane expressing agglutinin, and the response of Sesamia calamistis Hampson to Bt-maize expressing Cry 1Ab. Furthermore, Turlings et al. (2005) showed that Bt-maize emitted lower quantities of herbivore induced volatiles than a conventional maize. This may be important for host finding by the herbivorous insects. The setup in this study has a potential weakness, as the ovipositing females were raised on one of the hosts used in the experiment. This may lead to conditioning to this specific host (Hovanitz & Chang, 1962; Szentesi & Jermy, 1990), but in this study no conditioning was observed, as the females showed a profound oviposition preference for the other host plant (W45).

The experiments with larval food preference suggest that larvae are unable to distinguish between Bt and non-Bt host types. It was found that measurable consumption did occur on W45, despite the fact that significantly more was eaten from other plant varieties. These observations suggest that the larvae do not discriminate between the food sources, but that they stop feeding after a period. This is supported by the qualitative observation that larvae, upon placement in the experimental unit, moved around randomly and started to feed. If the food encountered was a W45, the larva would stop feeding after a short period of time (approximately 5 min) and lay still on the top of the leaf. Thereafter, their faeces turned reddish and, ultimately, they died. Other papers have shown that insertion of resistance genes may alter both oviposition and feeding behaviour of herbivorous insects (Alla et al., 2003; Bernal & Setamou, 2003; Rovenska et al., 2005; Sadeghi et al., 2006; Singh et al., 2008). We, therefore, find that an early assessment of acceptance/deterrence of the transgenic host plant, is both necessary for exposure assessment and might reduce the need of tests for risk assessment procedures in those examples, where transgenic varieties are repellent to herbivorous insects. In these cases, possible adverse effects are restricted to population effects due to less available food.

Food quality

The model species used in this study may encounter the transgenic plant at different stages of larval development, because it uses more plants over time. In such cases, it is relevant to evaluate the stage-specific response to the transgenic plant. It was found that all instars feeding on the transgenic W45 experienced 100% mortality. However, the time required to kill the larvae differed between instars, and increased with larval age (Figure 2). There may be various explanations for this differential larval response, but generally ecotoxicological studies show that early instars are more susceptible than later ones. For Bt-toxins this is exemplified in the studies of Hellmich et al. (2001), Kouassi et al. (2001), and Gilliland et al. (2002).

Population level assessment

The ecological question for the population model was: under which circumstances will the ecological consequences be severe? On this first simplistic level of effect assessment, population modelling is one approach to evaluate the probability of effects on a population scale and can guide the design of more elaborate testing. It is uncommon that butterflies produce so many eggs that the larvae have to disperse to other food plants to complete development. However, P. brassicae has been observed to lay egg clutches of up to 155 eggs per plant (Kristensen, 1994; Finch & Kienegger, 1997). Empirical data have also shown that this species uses more than one host. Davies & Gilbert (1985), for example, found that, for all egg clutches in their field experiment, the larvae defoliated their original host and dispersed to new plants. Not one of the original host plants was sufficient to support the entire larval development. Furthermore, Le Mausier (1991) observed that all larvae dispersed in their fourth or fifth instar.

The model exercise performed in this study showed that the number of plants necessary for complete development is important, as is the frequency of transgenic plants within subpopulations, whereas the significance of spatial distribution is negligible. These results, therefore, suggest that parameters that influence the number of plants needed for a whole life cycle should be described in larger detail at the next level of testing. Extra parameters could include, for example, predation level or preference among host species likely to coexist in nature. The importance of the frequency of transgenic plants within subpopulations also points out that a precise estimate of the likelihood of invasion of the transgenic plant into natural habitats, as well as the population structure of the host plants, is a prerequisite for a trustworthy risk assessment.

The presented model may also be used to suggest which types of habitats are relevant for monitoring the effects of insect-resistant plants on herbivorous insects. From Figure 3 it is obvious that the relevant frequency of resistant plants is dependent on the number of host plants necessary to complete larval development. Increasing b resulted in a lower probability of surviving to adulthood for the larvae. Differences were largest at a low frequency of resistant plants. The difference between fixed and stochastic (i.e., random or patchy) plant distributions is caused by the fact
that the number of host plants is close to the lower limit. By introducing variation in the number of plants within sub-populations, many larvae will experience food shortages, even when no toxic plants are present. However, this scenario is probably not relevant for this species, because Rothschild (1985) observed that the ovipositing female assesses the habitat rather than the single host plant on which the eggs are deposited.

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References


