

Bt maize pollen exposure and impact on the garden spider, *Araneus diadematus*

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Accepted: 28 September 2005

Key words: GMO, risk assessment, non-target organisms, Cry1Ab toxin, *Bacillus thuringiensis*, *Zea mays*, Araneae, Araneidae

Abstract

Concerns have been raised that Bt maize pollen may have adverse effects on non-target organisms; consequently, there is a general call for Bt maize risk assessment evaluating lethal and sublethal side effects. Spiders play an important economic and ecological role as pest predators in various crops, including maize. Web-building spiders, especially, may be exposed to the Cry1Ab toxin of Bt maize by the ingestion of pollen via 'recycling' of pollen-dusted webs and intentional pollen feeding. In this study, the potential Bt maize pollen exposure of orb-web spiders was quantified in maize fields and adjacent field margins, and laboratory experiments were conducted to evaluate the possible effects of Bt maize pollen consumption on juvenile garden spiders, *Araneus diadematus* (Clerck) (Araneae: Araneidae). In maize fields and neighbouring field margins, web-building spiders were exposed to high amounts of Bt maize pollen. However, a laboratory bioassay showed no effects of Bt maize pollen on weight increase, survival, moult frequency, reaction time, and various web variables of *A. diadematus*. A pyrethroid insecticide (Baythroid) application affected weight increase, survival, and reaction time of spiders negatively. In conclusion, the insecticide tested showed adverse effects on the garden spider, whereas the consumption of Bt maize pollen did not. This study is the first one on Bt maize effects on orb-web spiders, and additional research is recommended in order to account for further spider species, relative fitness parameters, prey-mediated effects, and possible long-term chronic consequences of Bt exposure.

Introduction

Genetically modified Bt maize commercially grown in Europe expresses the activated and truncated protein Cry1Ab of the soil bacterium *Bacillus thuringiensis* var. *kurstaki* (Berliner) in plant tissues, including pollen (Fearing et al., 1996). The Cry1Ab protein is described as specifically toxic against Lepidoptera, which include economically relevant pests in maize stands (Gill et al., 1992; Burkness et al., 2001). Due to this specificity, Bt maize is considered to be harmless to non-target organisms outside the order Lepidoptera (Glare & O'Callaghan, 2000; O'Callaghan et al., 2005), but there are indications that

invertebrate predators consuming Bt toxins or consuming prey that had fed on Bt toxins may be affected adversely (Hilbeck et al., 1998a,b, 1999; Dutton et al., 2002). However, the latter results conflict with other studies reporting no negative impact, and there is an ongoing debate as to whether recorded negative effects of Cry1Ab are through indirect bitrophic and tritrophic pathways rather than direct effects of the Bt toxin itself (Pilcher et al., 1997; Andow & Hilbeck, 2004; Romeis et al., 2004). In general, laboratory data on the impact of Bt maize toxin on invertebrate natural enemies are limited (Lövei & Arpaia, 2005), and there is a common need and call for Bt maize risk assessment (e.g., European Parliament & Council, 2001; Züghart & Breckling, 2003). Criteria for the choice of indicator species as test organisms for a Bt maize risk assessment should include ecological and/or economic significance of the organisms in (agro-)ecosystems, existing exposure pathways, the degree of the exposure of the organisms to the genetically modified organism (GMO), as well as acute and chronic toxicity of the transgenic

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product (Jepson et al., 1994; Hilbeck & Andow, 2002; Dutton et al., 2003; Andow & Hilbeck, 2004).

Spiders are abundant invertebrate predators in arable land, belong to the dominant predators in maize fields, and play an important economic and ecological role as natural enemies in various crops, including maize (Lang et al., 1999; Sunderland, 1999; Albajes et al., 2003; Lang, 2003; Nyffeler & Sunderland, 2003; Candolfi et al., 2004; Ludy & Lang, 2004). Spiders, especially web-building spiders, may be exposed to the Cry1Ab toxin of Bt maize through different exposure routes. Bt maize pollen is dispersed by wind (Lang et al., 2004) and may be collected by spider webs inside and outside maize fields (Bera et al., 2002; Ludy, 2004). By recycling their webs, orb-web spiders consume pollen adhering to the sticky web spiral, which may even be an essential additional food source for juveniles (Smith & Mommsen, 1984; Ludy, 2004), whereas hunting spiders without a web may actively forage for the pollen (Vogelei & Greissl, 1989). Furthermore, spiders may take up toxin by preying on herbivorous prey that has fed on Bt maize tissue (cf. Dutton et al., 2002), or catch maize pollen-collecting insects. Accordingly, the presence of Cry1Ab endotoxins was recorded in over 7% of spiders collected in a transgenic Bt maize field, indicating a long-term exposure to insecticidal Bt toxins (Harwood et al., 2005).

Surprisingly, there has not been one laboratory experiment testing the impact of Bt maize on spiders, nor are there studies quantifying the field exposure of spiders to the toxin of transgenic Bt maize apart from the recently published analysis of Harwood et al. (2005), despite the ecological and economic significance of spiders and their potential exposure to Bt toxin (Lövei & Arpaia, 2005). Behavioural parameters of indicator species may be more sensitive markers of a toxic effect than sheer mortality. Orb webs of spiders are true and direct reflections of the spiders' complex web-building behaviour (e.g., Vollrath, 1986, 1992), and orb-web geometry is affected by and does indicate the exposure to pesticides (Samu & Vollrath, 1992; Lengwiler & Benz, 1994; Hesselberg & Vollrath, 2004).

Alteration of web structure patterns may even have an ecological effect, as prey capture efficacy is influenced by web geometry (Eberhard, 1986).

The garden spider, *Araneus diadematus* (Clerck) (Araneae: Araneidae), is a common (pest) predator in various habitats neighbouring arable fields (Hänggi et al., 1995); it occurs also in maize fields (Nyffeler, 1982) and can be easily kept in the laboratory (Zschokke & Herberstein, 2005), all of which makes it an ideal 'model spider' for studying Bt maize effects. In this study, we quantified potential Bt maize pollen exposure of orb-web spiders in the field by recording the number of maize pollen grains found in orb webs located in maize fields and adjacent margins. The effect of Bt maize pollen consumption on lethal and sublethal parameters, including web-building behaviour, was studied in the laboratory by applying Bt pollen to the webs of juvenile *A. diadematus*.

Materials and methods

Potential field exposure of orb-web spiders to Bt maize pollen

For the study of potential Bt maize pollen exposure, orb-web-building spiders were kept in the laboratory. Here, they could build their webs within wooden frames (Figure 1A). These webs were then exposed for a standardized time period within and at the edge of pollen-shedding Bt maize fields (Figure 1B), and subsequently, numbers of maize pollen grains deposited in the webs were analysed. In general, by using the term 'exposure', we refer to potential exposure of spiders to pollen and it is not implied that 'potential exposure' necessarily equals 'effective exposure', the latter implying the intake of a substance (Moriarty, 1988).

Obtaining spider webs. Field-collected adult females of various orb-web spider species of the family Araneidae [*Araneus diadematus* (Clerck), *Argiope bruennichi* (Scopoli), *Aculepeira ceropegia* (Walckenaer), and *Larinioides* species (Caporiacco)] were used for the investigation of horizontal



Figure 1 Wooden frame covered with two plastic plates for keeping orb-web spiders in the laboratory (A) and the exposure of spider webs at different distances from a maize field to determine pollen amounts in webs (B).

distribution and vertical dispersion of Bt maize pollen in orb webs. Gross web parameters of araneid species are similar, and species-specificity is uncommon (Eberhard, 1990). Spiders were allowed to build their webs within wooden frames of 30 × 30 cm (Figure 1A). Two transparent plastic plates enclosed both open sides of the frames to prevent spiders from escaping, the plates being covered on the inside with vaseline to prevent spiders from attaching silk threads to the plate (Hesselberg & Vollrath, 2004; Zschokke & Herberstein, 2005). The spiders were supplied with *Drosophila* flies and watered every day. Prior to the exposure of spider webs to maize pollen in the field, spiders were carefully removed from their webs with a small brush, because the webs were exposed in the field without the spiders. This was done to prevent a destruction of the web by the spider, and to prevent the loss of escaping spiders, which were needed and used to build new webs in the laboratory.

Exposure of spider webs in the field. Bt maize pollen numbers deposited in spider webs were investigated in July 2002 during maize anthesis on two state research farms in Upper Bavaria and Swabia (south Germany). In Upper Bavaria, a Bt maize field of 30 × 50 m and in Swabia, a Bt maize field of 2 ha were planted (at both locations maize event Bt176, cultivar 'Navares' from Syngenta, Basel, Switzerland). The event Bt176 is one of the two events being currently registered for the cultivation of Bt maize in Europe, the other one being MON810. In the smaller field (30 × 50 m), the vertical distribution of maize pollen was studied within the field (i.e., different heights of spider webs). In the larger field (2 ha), the horizontal distribution of maize pollen was studied on the northern field margin (i.e., different distances of webs to field edge).

For the investigation of the vertical distribution of Bt maize pollen, frames with spider webs (but without the spider) were mounted at different heights of 20, 80, and 170 cm in the middle of the pollen-shedding Bt maize field (BBCH growth stadium 6.5, i.e., 'upper and lower parts of tassel in flower'; Meier, 1997). Each height was replicated three times on four sampling occasions between 28 July and 31 July, 2002, resulting in a total of 36 webs (3 heights × 3 replicates × 4 dates). Each web was exposed for 24 h, which was considered to be a realistic exposure time, as spiders were reported to recycle their webs once a day (Breed et al., 1964). During this investigation, the weather conditions were 23.2 ± 2.2 °C for air temperature, 0.83 ± 0.43 m s⁻¹ for wind velocity (arithmetic mean of daily means ± SD), and 5.95 ± 11.83 mm for precipitation (arithmetic mean of daily sums ± SD).

For the analysis of the horizontal dispersion of Bt maize pollen, spider webs were exposed at a height of 80 cm and 5 m within the field (-5 m), as well as at distances of 0

(maize field edge), 3, 6, and 10 m to the maize field in the adjacent field margin (Figure 1B). On this field margin, natural succession of the vegetation community was allowed to grow (Figure 1B). The Bt maize field was in full anthesis (BBCH growth stadium 6.5; Meier, 1997). The experiment took place on five sampling occasions between 18 July and 24 July, 2002. Each distance was replicated four times per day, resulting in a total of 100 webs (5 distances × 4 replicates × 5 dates). As the exposed webs were frequently destructed by wind or moving plant material, only 53 webs out of 100 could be analysed. Again, webs were exposed for a 24-h period per sampling date. This field assay was conducted under the following weather conditions (arithmetic means of daily means ± 1 SD): air temperature of 15.5 ± 2.4 °C, wind velocity of 1.40 ± 0.59 m s⁻¹, and precipitation (arithmetic mean of daily sums ± SD) of 4.36 ± 7.62 mm.

After field exposure, frames with spider webs were sealed on both sides with plastic plates for transportation. In the laboratory, pollen-loaded webs were photographed with a digital camera against a black background. Pollen grain numbers in spider webs were counted manually from enlarged paper copies of the digital pictures. The reliability of the pollen counting approach was evaluated by comparing pollen numbers counted directly from spider webs by eye, with pollen counted from enlarged paper copies of the referring webs. The regression equation was 'Pollen counts from paper copies' (n) = 0.434 + 0.971 × 'Directly counted pollen' (n) (R² = 0.93, P < 0.001; n = 53).

Statistical analysis. Linear regression analyses were calculated to analyse the amount of maize pollen in spider webs related to 'height in maize field' (cm) and 'distance from maize field edge' (m), respectively. In addition, a multiple regression with backward selection was applied to estimate the influence of the independent variables, 'area of spider webs (cm²)', 'air temperature (°C)' and 'wind velocity (m s⁻¹)' (both daily means), 'precipitation (mm)' (daily sums), on the number of pollen in webs. The probability of F -to remove was 0.10. When necessary, data were log or log(x + 1)-transformed to meet the assumptions of linear regression.

Uptake of the Cry1Ab protein by spiders

Spiders ingest Bt maize pollen and take up Cry1Ab protein (Ludy, 2004). To quantify the amount of Cry1Ab that was ingested by spiders while pollen feeding, an enzyme-linked immunosorbent assay (ELISA) was conducted. For this purpose, 20 juvenile garden spiders (*A. diadematus*) were kept under standardized conditions (temperature 20 °C, L10:D14 regime) in wooden frames (10 × 10 cm) in the laboratory and were fed with fruit flies daily. Bt maize

pollen (cultivar 'Navares' from Syngenta) was collected in July 2002 from fields in Frankonia and Swabia, south Germany, located on experimental farms of the Bavarian State Research Center for Agriculture (LfL, 2005). Obtained pollen was frozen and stored at -18°C for 10 months prior to the tests. Before use in the laboratory tests, pollen was defrosted and desiccated in a drying chamber at 30°C for 2 days and was then sieved through a 0.1-mm mesh. After the spiders had built webs regularly, webs were dusted with Bt maize pollen (cultivar 'Navares', Syngenta). Right after the spiders had recycled their webs, the spiders were frozen at -18°C and stored for 6 months. Then spiders were defrosted at 5°C and washed with water to remove pollen possibly adhering to the spider. Additionally, spiders were checked carefully for adhering pollen under a binocular microscope. Subsequently, the spiders' gastrointestinal systems were dissected and taken up in cyclohexylamino-propane sulfonic acid buffer (CAPS buffer: 50 mM, pH 10.5). The dissected tissue was analysed for Cry1Ab content with a commercial ELISA kit (EnviroLogix QuantiPlate™ kit for Cry1Ab/Cry1Ac from Neogen Europe Ltd, Auchincruive, UK). Also, the Cry1Ab content of the applied Bt maize pollen was quantified with the ELISA kit. The limit of detection of the kit was 0.14 p.p.b.; however, the limit of quantification of Cry1Ab was 0.50 p.p.b.

Test of biological activity of the Cry1Ab protein

The biological activity of the Cry1Ab in Bt maize pollen used in the spider laboratory assay was tested on a target organism of Bt maize, the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae). Pollen was obtained and processed as described in 'Uptake of the Cry1Ab protein by spiders' (see previous discussion). L1-L2 larvae of *O. nubilalis* were kept in 24-well tissue culture plates with one larva per well. Wells were filled with 0.5 ml of an artificial diet with 1% conventional maize pollen ($n = 18$), 1% Bt maize pollen ($n = 15$), or without pollen ($n = 17$), respectively. Due to a shortage of pollen, we applied an amount of Bt pollen that was below the LC_{50} for *O. nubilalis* (Meise, 2003). Mortality of the larvae was registered each day after the start of the bioassay for 7 days. ANOVA was used to analyse the effect of the factor 'diet' (i.e., diet with Bt maize pollen, with conventional maize pollen, and without pollen) on the dependent variable 'survival' (days). Larvae that survived the whole observation time of the experiment (7 days) were set to the survival time of 7 days. Least significant differences (LSD) test was conducted for multiple comparisons between diets.

Effects of Bt maize pollen on *Araneus diadematus*

The laboratory assays to study the effect of Bt maize pollen consumption on orb-web spiders were carried out with

juvenile garden spiders (*A. diadematus*). The spiders were obtained from cocoons made by field-collected female garden spiders. The spiderlings were kept in wooden frames (10×10 cm) and provided with *Drosophila* flies and watered every second day. About 5 weeks after hatching, a sufficient number of spiders built small webs in the frames regularly; these were then used for the laboratory assay.

Experimental procedure. Spiders were fed with Bt maize pollen; one control group was given conventional maize pollen, and a second control was treated with an insecticide. Bt maize pollen (cultivar 'Navares' from Syngenta) and conventional maize pollen (near-isogenic cultivar 'Antares', Syngenta) were obtained and processed as described in 'Uptake of the Cry1Ab protein by spiders' (see previous discussion). The insecticide used was the pyrethroid Baythroid 50® from Bayer AG, Leverkusen, Germany (active ingredient Cyfluthrin) and was applied in a concentration of $2 \mu\text{l l}^{-1}$. As we intended to study sublethal effects of Bt maize pollen and Baythroid 50® on web parameters (see succeeding discussions), it was crucial that enough spiders would survive. A relevant field dose of a pyrethroid insecticide (Samu et al., 1992), however, would have been lethal for juvenile *A. diadematus* (Samu & Vollrath, 1992). Considering that we needed enough spiders alive for recording web-building parameters, we calculated and applied a dose referring to $0.8 \mu\text{g}$ Cyfluthrin per gram spider, which is below the LD_{50} of $2.2 \mu\text{g g}^{-1}$ spider for Cypermethrin, the active ingredient of another pyrethroid (Samu & Vollrath, 1992).

Out of the total of 60 spiders that built webs in wooden frames, spiders were assigned randomly to the three different treatments: conventional maize pollen, Bt maize pollen, and the insecticide Baythroid. Prior to the assay, all spiders were weighed ('initial weight') and were fed 1–2 *Drosophila* flies daily during the experiment. For pollen application to a spider web, the wooden frame was carefully rotated by 90° together with the web and the spider. Then, pollen was applied over the capture spiral of the web from above with the help of a small pollen-loaded brush. Webs of spiders in the pollen treatments were additionally sprayed daily with water using a fine sprayer. Webs of insecticide-treated spiders were sprayed with a water-based Baythroid solution ($2 \mu\text{l l}^{-1}$) instead of pure water. Webs were sprayed until they were satiated with fluid, and the applied Baythroid amount was calculated from the area and the density of the sticky spiral of the treated webs according to a general water absorbance estimation of spider webs (Samu & Vollrath, 1992).

The construction of new webs was controlled daily, and all treatments (pollen and insecticide) were applied to four webs built by each spider in order to standardize

pollen and insecticide amount. This approach meant that the treatment duration differed among spiders, depending on the web-building frequency of individual spiders.

Data recording and processing. After each pollen application, the spider web was photographed with a digital camera to determine the amount of pollen in the web. The webs were photographed using an illuminated and velvet-lined box (described in Zschokke, 2002). Pollen grains in spider webs were counted manually from enlarged paper copies of the digital pictures. During the experiment, every feeding of the spiders was recorded by a digital video camera to record the reaction time of the spider to prey. The reaction time was defined as the time the spider needed to respond to a fruit fly, i.e., the time span between the time the fly was caught in the web and the first movement of the spider towards the fly. As flies sometimes rested motionless when get stuck in the spider web, reaction times of the spider were prolonged by such a fly behaviour. Therefore, only cases with reaction times <1 s were included into the analysis to prevent a bias due to fly behaviour. Reaction-time analysing was carried out with the program CYBERLINK POWERDIRECTOR™ 1.1 (CyberLink Corp., Taipei, Taiwan). Mortality and moulting of the spider (if occurring) were recorded daily until the last spider died. The first web that spiders built after the last pollen or insecticide application was photographed with a digital camera and the spider was weighed at this date ('end weight'). After treatment, the length of the tibia of the first right leg was recorded by measuring exuviae or dead spiders with a stereo microscope and an ocular micrometer. Possible 'web-building inhibition' was recorded and defined as the time span that the spider took to build a new web after the last treatment application. Various web parameters of the first web after treatment were analysed with the image-analysing program SCION IMAGE FOR WINDOWS 4.0 (Scion Corp., Frederick, MD, USA): length of radii (cm), length (cm), and area (cm²) of the sticky spiral, as well as density of the sticky spiral (length of sticky spiral divided by the area of sticky spiral (cm cm⁻²)) (see Zschokke, 1999 for web nomenclature).

Statistical analysis. Analysis of covariance (ANCOVA) was used to analyse the effect of the main factor 'treatment' (i.e., conventional maize pollen, Bt maize pollen, and Baythroid) on the recorded dependent variables: spider weight increase ('end weight' minus 'initial weight', mg), survival (days), moult frequency (numbers of moults in 20 days), 'web-building inhibition' (days), and reaction time (ms, means of measures during treatment). For all analyses, except for the dependent variable 'reaction time', the covariate 'treatment duration' (time between the first

and the last treatment application) was included, because treatment duration differed among spiders (see previous discussion). Likewise, treatment effects on web geometry were tested with ANCOVA: length of radii (cm), length of sticky spiral (cm), area of sticky spiral (cm²), and the density of the sticky spiral (cm cm⁻² spider web). In all analyses of web geometry variables, the covariates 'length of tibia' and 'treatment duration' were included. The covariate 'length of tibia' was considered because the size of a spider web is influenced by the leg length of spiders (Vollrath, 1987).

All variables and covariates were log(x + 1)-transformed to create normal distribution and/or homogeneity of variances. LSD test was applied for pairwise comparisons. Furthermore, observed and standardized effect sizes (Cohen's d) and the corresponding 95% confidence intervals were calculated from estimated means for pairwise comparisons of each dependent variable (Nakagawa & Foster, 2004) following a SPSS script described in Smithson (2001), which also gives an indication of the statistical power to detect differences between treatments. The SPSS script can be downloaded from the Internet at <http://www.anu.edu.au/psychology/people/smithson/details/CIstuff/CI.html>. All statistical analyses were carried out using SPSS, version 11 (SPSS Inc., Chicago, IL, USA). All average values presented are arithmetic means ± SD, and all tests are two-sided.

Results

Bt maize pollen loads in spider webs

In the maize field, the average pollen number per spider web was 1044 ± 1193 (95% CI: 698–1391). Exposed spider webs had a mean area of the sticky spiral of 197.35 ± 198.58 cm², resulting in an average of 6.87 ± 6.05 pollen grains per cm² web area. There was a positive relationship between the pollen load in spider webs and the height of their position in the maize field (R² = 0.29, P < 0.01; Figure 2). Other variables were contributing to the variance in pollen numbers according to the regression equation: pollen amount in spider web (n) = 3080.02 + 1241.96* Log₁₀(height in maize field) + 294*sticky spiral area – 202.85* air temperature (R² = 0.46, P < 0.01).

In the field margin neighbouring the Bt maize field, the mean pollen number found per spider web was 381 ± 205 (95% CI: 313–450). Exposed spider webs had a mean area of the sticky spiral of 187.49 ± 97.64 cm² resulting in an average number of 2.55 ± 2.07 pollen grains per cm² web area. There was a negative relationship between the pollen amount in spider webs and the distance to the maize field (R² = 0.12, P = 0.01; Figure 3). Other variables were contributing to the variance in pollen numbers according to the regression equation: pollen amount in spider web

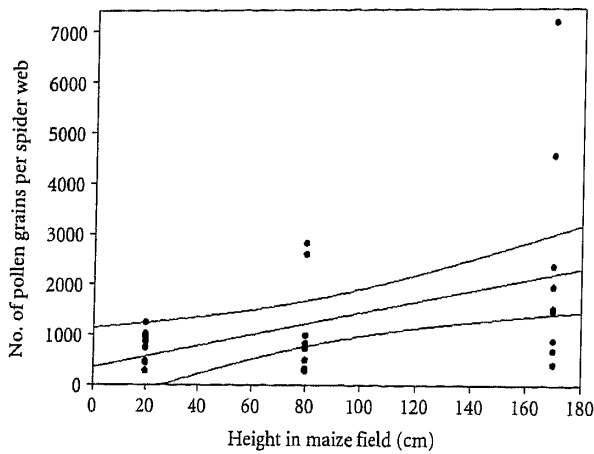


Figure 2 Number of Bt maize pollen recorded in webs of Araneidae related to the vertical position of the spider webs (height) in the maize field. The regression equation is $y = 361.11 + 10.72x$, $R^2 = 0.29$, $P = 0.002$, $n = 32$ (linear regression is given with the upper and lower limit of the 95% confidence interval).

($n = 1927.06 - 14.88 \times \text{distance} - 63.51 \times \text{air temperature} - 370.14 \times \text{wind speed} + 9.26 \times \text{precipitation}$ ($R^2 = 0.38$, $P < 0.001$).

Uptake of the Cry1Ab protein by spiders

The Cry1Ab protein was detected in the gastrointestinal system of 13 out of 20 garden spiders (65%), whose webs were treated with Bt maize pollen. The applied Bt maize pollen had a mean Cry1Ab concentration of 2657.81 ± 537.34 p.p.b. The Cry1Ab amount in the gastrointestinal system of spiders was < 0.5 p.p.b.

Test of biological activity of the Cry1Ab protein

Diet had an effect on the survival time of *O. nubilalis* larvae (ANOVA: $F_{2,47} = 8.67$, $P = 0.001$), and the Bt pollen was biologically active. Mean survival times of *O. nubilalis* larvae fed with conventional maize pollen was 6.94 ± 0.24

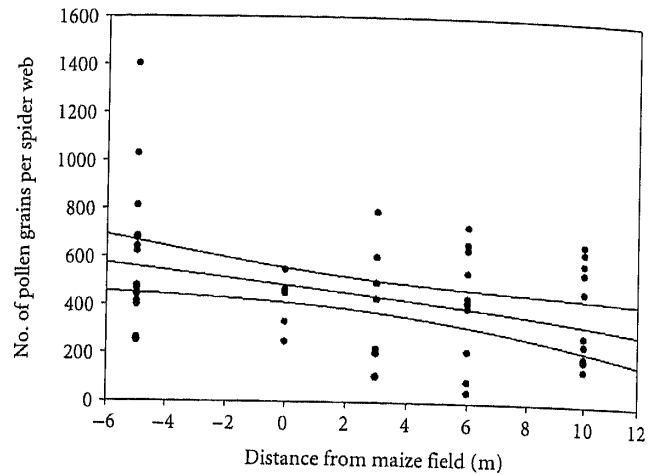


Figure 3 Number of Bt maize pollen recorded in webs of Araneidae related to the distance to the maize field. The regression equation is $y = 483.81 - 15.16x$, $R^2 = 0.12$, $P = 0.01$, $n = 53$ (linear regression is given with the upper and lower limit of the 95% confidence interval).

days ($n = 18$), 5.00 ± 2.27 days ($n = 15$) when fed with Bt maize pollen, and 6.71 ± 1.21 days ($n = 17$) in the group on a diet without pollen. Survival time was significantly lower for Bt maize pollen fed larvae as compared to the conventional maize pollen treatment ($P = 0.002$), or larvae without any pollen ($P < 0.001$, LSD test in both cases). After 7 days, 53% of the larvae fed a diet with Bt maize pollen had died, whereas mortality of larvae on a diet without Bt maize pollen was only 6%.

Effect of Bt maize pollen and insecticide on *Araneus diadematus*

Treatment conditions and characteristics of test spiders can be obtained from Table 1. Treatment showed an effect on spider variables 'weight increase' ($F_{2,39} = 5.03$, $P = 0.01$), survival ($F_{2,36} = 7.74$, $P = 0.002$), and reaction time ($F_{2,37} = 2.90$, $P = 0.07$), but not on moult frequency ($F_{2,35} = 0.38$, $P = 0.69$) (ANCOVA in all cases, Figure 4). The

Table 1 Treatment conditions and characteristics of *Araneus diadematus* treated with conventional maize pollen, Bt maize pollen, and the insecticide Baythroid (arithmetic means \pm SD)

| | Conventional maize pollen | Bt maize pollen | Insecticide |
|--|---------------------------|----------------------|------------------------------|
| Applied pollen numbers per web (number) | 871.01 \pm 536.14 | 1177.56 \pm 902.16 | No pollen |
| Applied Baythroid 50 amount per web (nl) | No insecticide | No insecticide | 0.05 \pm 0.01 ^a |
| Treatment duration (days) | 10.00 \pm 3.59 | 10.36 \pm 3.72 | 10.38 \pm 3.64 |
| Tibia length (mm) | 1.61 \pm 0.26 | 1.48 \pm 0.38 | 1.49 \pm 0.61 |
| Initial spider weight (mg) | 3.04 \pm 2.01 | 2.79 \pm 1.88 | 3.58 \pm 2.23 |
| Final spider weight (mg) | 6.31 \pm 2.40 | 5.79 \pm 2.43 | 5.97 \pm 3.43 |
| Age of spiders (days) | 42.56 \pm 1.85 | 42.43 \pm 2.06 | 42.14 \pm 1.79 |

^aBaythroid amount was estimated from the area and the density of the sticky spiral of the treated webs and the estimated water absorbance after Samu & Vollrath (1992).

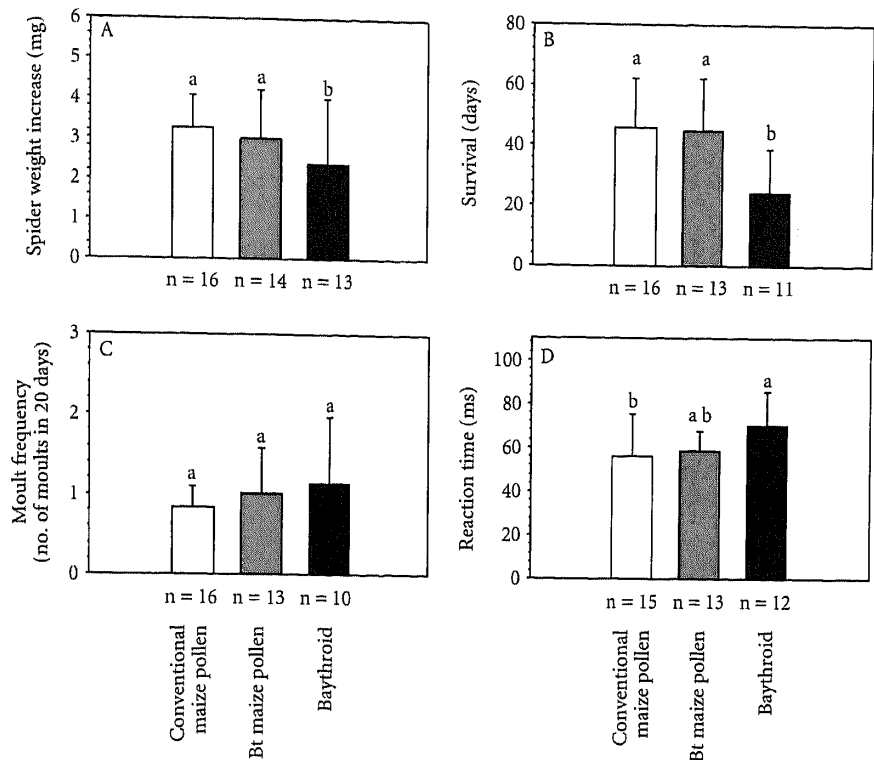


Figure 4 (A) Weight increase, (B) survival, (C) moult frequency, and (D) reaction time towards prey of *Araneus diadematus* spiders in the laboratory treated with conventional maize pollen, Bt maize pollen and the insecticide Baythroid (arithmetic means + SD). Columns capped with the same letter do not differ significantly (LSD test, P > 0.05).

covariate 'treatment duration' had a positive effect on 'weight increase' ($F_{1,39} = 12.29, P = 0.001$), but no relationship with the variables 'survival time' ($F_{1,36} = 2.34, P = 0.13$) and 'moult frequency' ($F_{1,35} = 0.23, P = 0.64$).

There was no difference between conventional and Bt maize pollen-treated spiders concerning spider weight increase (Figure 4A), survival (Figure 4B), moult frequency (Figure 4C), and reaction time (Figure 4D) (LSD test in all cases, Table 2). However, Baythroid-treated spiders had a reduced weight increase, a lower survival, and a longer reaction time (Figure 4, Table 2).

Treatment did not cause web-building inhibition ($F_{2,35} = 0.28, P = 0.76$): after termination of the treatment, spiders treated with conventional maize pollen built their first web after 2.00 ± 2.24 days, Bt maize-treated spiders after 2.09 ± 1.92 days, and Baythroid-treated spiders after 1.46 ± 0.66 days. The covariate 'treatment duration' had no influence on 'web inhibition' ($F_{1,35} = 0.37, P = 0.55$).

There was no treatment effect recorded on any of the web variables (Figure 5, ANCOVA in all cases): 'length of radii' ($F_{2,34} = 0.38, P = 0.86$), 'length of sticky spiral' ($F_{2,34} = 0.14, P = 0.87$), 'area of sticky spiral' ($F_{2,34} = 0.12,$

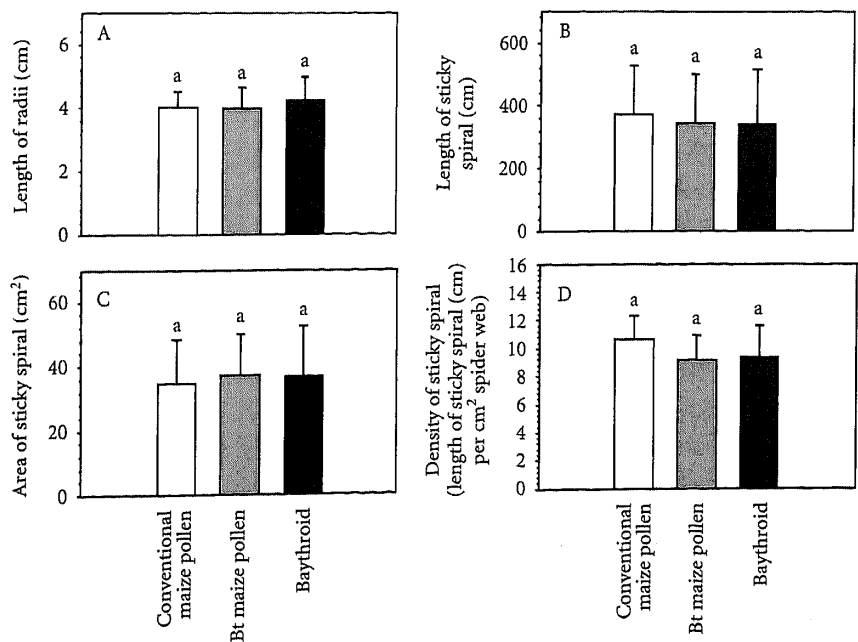


Figure 5 (A) Length of radii, (B) length of sticky spiral, (C) area of sticky spiral, and (D) density of sticky spiral of *Araneus diadematus* spiders in the laboratory treated with conventional maize pollen, Bt maize pollen, and the insecticide Baythroid (arithmetic means + SD). Sample sizes are 15 spiders for conventional pollen, 11 spiders for Bt maize pollen, and 13 spiders for Baythroid treatment. Columns capped with the same letter do not differ significantly (LSD test, P > 0.05).

| Comparison | P value | d obs. | Cohen's d | 95% CI of d |
|--|---------|--------|-----------|-------------|
| Weight increase (mg) | | | | |
| Conventional–Bt | 0.44 | 0.25 | 0.14 | –0.44–1.00 |
| Conventional–insecticide | <0.01 | 0.98 | 0.58 | 0.37–1.96 |
| Bt–insecticide | 0.03 | 0.73 | 0.45 | 0.08–1.68 |
| Survival (days) | | | | |
| Conventional–Bt | 0.99 | 0.55 | <0.01 | –0.19–0.19 |
| Conventional–insecticide | <0.01 | 21.25 | 0.72 | 0.58–2.32 |
| Bt–insecticide | <0.01 | 20.70 | 0.72 | 0.52–2.33 |
| Moult frequency (number per 20 days) | | | | |
| Conventional–Bt | 0.42 | –0.04 | 0.15 | –0.43–1.04 |
| Conventional–insecticide | 0.56 | –0.03 | 0.12 | –0.56–1.03 |
| Bt–insecticide | 0.87 | 0.01 | 0.03 | –0.72–0.86 |
| Reaction time (ms) | | | | |
| Conventional–Bt | 0.41 | –2.44 | 0.16 | –0.43–1.06 |
| Conventional–insecticide | 0.02 | –13.90 | 0.48 | 0.15–1.76 |
| Bt–insecticide | 0.14 | –11.46 | 0.31 | –0.19–1.36 |
| Length of radii (cm) | | | | |
| Conventional–Bt | 0.96 | –0.15 | 0.01 | –0.76–0.80 |
| Conventional–insecticide | 0.46 | –0.23 | 0.14 | –0.49–1.07 |
| Bt–insecticide | 0.45 | –0.22 | 0.16 | –0.47–1.12 |
| Length of sticky spiral (cm) | | | | |
| Conventional–Bt | 0.86 | 10.43 | 0.04 | –0.71–0.85 |
| Conventional–insecticide | 0.60 | 16.49 | 0.10 | –0.55–0.94 |
| Bt–insecticide | 0.75 | 6.06 | 0.07 | –0.67–0.93 |
| Area of sticky spiral (cm²) | | | | |
| Conventional–Bt | 0.63 | –3.38 | 0.10 | –0.59–0.97 |
| Conventional–insecticide | 0.87 | –3.02 | 0.03 | –0.68–0.81 |
| Bt–insecticide | 0.74 | 0.36 | 0.07 | –0.67–0.94 |
| Density of sticky spiral (cm cm⁻²) | | | | |
| Conventional–Bt | 0.13 | 1.35 | 0.31 | –0.18–1.41 |
| Conventional–insecticide | 0.12 | 1.23 | 0.30 | –0.16–1.36 |
| Bt–insecticide | 0.98 | –0.12 | 0.01 | –0.42–0.43 |

Table 2 P values, observed (d obs.), and standardized effect sizes (Cohen's d) with the corresponding 95% confidence intervals for Cohen's d (95% CI of d) for pairwise comparisons (LSD test) of various dependent variables

$P = 0.88$), and 'density of sticky spiral' ($F_{2,34} = 1.67$, $P = 0.20$). None of the covariates 'treatment duration' and 'length of tibia' had an influence on web variables (variables: 'length of radii': $F_{1,34} = 0.05$, $P = 0.83$; $F_{1,34} = 1.19$, $P = 0.28$; 'length of sticky spiral': $F_{1,34} = 0.67$, $P = 0.42$; $F_{1,34} = 1.47$, $P = 0.23$; 'area of sticky spiral': $F_{1,34} = 0.36$, $P = 0.56$; $F_{1,34} = 0.32$, $P = 0.57$; 'density of sticky spiral': $F_{1,34} = 0.43$, $P = 0.51$; $F_{1,34} = 2.54$, $P = 0.12$). Observed and standardized effect sizes (Cohen's d) and corresponding 95% confidence intervals for pairwise comparisons of each dependent variable are shown in Table 2.

Discussion

Potential exposure of spiders to Bt maize pollen in the field

This study demonstrated that orb-web spiders are potentially exposed to maize pollen, both in the maize field and on its margin. Potential exposure to Bt maize pollen

could be very high, but at the same time very variable. However, it is unclear how much of the pollen spiders will take up. Spider webs positioned higher in the maize field received a larger pollen amount, which is in accordance with higher concentrations of airborne maize pollen in 2 m, as compared to 20 cm above ground (Jarosz et al., 2003). Spider webs on field margins contained less pollen than webs within maize fields. Following maize pollen dispersion on maize field margins (Lang et al., 2004), webs at a distance of 10 m to the maize field edge had only half as much pollen as webs located directly in or at the maize field. Web spider species and individuals building their webs at a higher position and/or closer to a Bt maize field would therefore be clearly more exposed to Bt maize pollen. Web position, distance of the web to the maize field, and pollen deposited by shedding maize could explain only a small proportion of the pollen load in webs. Other (meteorological) factors may contribute to the variance of

pollen amounts in spider webs such as temperature, turbulences, thermal convections, or wind speed, wind direction, and precipitation (e.g., Feil & Schmidt, 2001; Jarosz et al., 2003; Lang et al., 2004). Features of the spider webs themselves possibly contribute to the variance of pollen load. For instance, the stickiness of spider webs is influenced by temperature, probably due to desiccation of the sticky spiral glue (Edmonds & Vollrath, 1992). Furthermore, strong wind or insects caught in the web may cause single silk threads to merge, thus decreasing the web's overall catching area (C Ludy, pers. obs.). Preying on and catching pollen-collecting bees may result in even higher Bt maize pollen exposure of orb-web spiders (Hirschfelder, 1950; Ibrahim & Selim, 1972) and would warrant further investigation.

Orb-web spiders are not only exposed to maize pollen, they also feed on it. As maize pollen has a width of 90 μm (Aylor, 2002), it is unlikely that spiders, especially juveniles, consume pollen grains as a whole, because spiders are generally only able to ingest particles $\leq 1 \mu\text{m}$ (Foelix, 1992). Therefore, spiders would consume maize pollen by external digestion through various digestive enzymes and suck up the dissolved nutrients. Nevertheless, the Cry1Ab protein (or derivatives) could be detected in garden spiders that had recycled their Bt maize pollen-loaded webs. Spiders not only consume pollen accidentally when recycling their webs (Smith & Mommsen, 1984), but also utilize and feed on pollen intentionally, and may use it as an additional food source (Vogelei & Greissl, 1989; Ludy, 2004). The fact that only in 65% of the spiders was Cry1Ab detected may be due to Bt concentrations below the limits of detection, or may indicate that spiders do not always consume the pollen in the web.

Effect of Bt maize pollen on spiders. Bt maize pollen consumption had no detectable effect on juvenile garden spiders (*A. diadematus*) as compared to the consumption of conventional maize pollen. The lethal and sublethal parameters of spiders studied were unaffected, as were the recorded web variables. It has to be noted that we used pollen from the Bt maize event Bt176, which has a toxin content several times higher than pollen from the event MON810 (e.g., Lang et al., 2004), the other Bt maize event registered in Europe; therefore, exposure by MON810 would even be lower. This would also correspond to field studies that have not found adverse effects of Bt maize on the abundance of spiders (Hassell & Shepard, 2002; Jasinski et al., 2003). In this study, applied Bt maize pollen densities corresponded to the actual field situation, spiders took up the Bt toxin, and the Bt toxin of the pollen was still biologically active. It is acknowledged, however, that high variation and small effect sizes of the data of the Bt

comparison resulted in low power to detect effects, and that an existing Bt effect may have been missed due to limited sample size. In particular, web designs of individual spiders are highly variable (Hesselberg & Vollrath, 2004), which may be true especially for juvenile spiders (Heiling & Herberstein, 2000). Therefore, more mature or adult spiders have been suggested as indicator organisms (Witt & Reed, 1965). However, we chose, and still recommend, taking juvenile spiders, because the majority of the spider community in the maize fields during anthesis are juvenile stages (Ludy & Lang, 2004; Meissle & Lang, 2005).

The insecticide Baythroid caused clear adverse effects on spiders, proving that the experimental design was appropriate to detect effects. In the case of significant results, effect sizes were much larger than for the comparisons Bt vs. conventional pollen, resulting in a much higher power to statistically prove differences with the given sample size (cf. Meissle & Lang, 2005). Survival and weight increase of insecticide-treated spiders were reduced. Furthermore, pyrethroids can lead to a reduced mobility of spiders (Jagers op Akkerhuis et al., 1997), which may be responsible for the recorded longer reaction times of Baythroid-treated garden spiders. This negative impact of a pyrethroid insecticide confirms several other laboratory and field studies (e.g., Pekár, 2002; Meissle & Lang, 2005). In addition to the negative effect of the insecticide itself, the pollen treatments received an additional protein supply through the maize pollen, which might have contributed to differences in survival and weight increase. In contrast to the present study, several other studies found an impact of insecticides on web geometry (Samu & Vollrath, 1992; Lengwiler & Benz, 1994). In this study, the applied Baythroid concentration was relatively low in order to prevent an early loss of test spiders. As mentioned previously, the high variability of web parameters possibly obstructed the detection of an effect. Moreover, in studies demonstrating a negative pesticide effect on web-geometry parameters, pesticides were mostly applied orally or topically (e.g., Samu & Vollrath, 1992; Lengwiler & Benz, 1994), which probably results in higher doses compared to insecticide-sprayed webs.

Conclusions

Web-building spiders are exposed to Bt maize pollen in maize fields and adjacent field margins. Most likely, orb-web spiders will feed on the Bt maize pollen caught in their webs, but the laboratory assay showed no adverse effects of Bt maize pollen consumption on the garden spider, *A. diadematus*, whereas an insecticide had a clear negative impact. Spiders are common and abundant predators in arable land important for pest control, and this study, together with the so far published field results, suggests

that cultivation of Bt maize is more compatible with biological control provided by spiders than the application of a pyrethroid insecticide. However, this is the first study investigating the potential exposure and effects of Bt maize on an orb-web spider in the laboratory. Therefore, the database is still too small to allow for generalizations. Considering the important ecological and economic role of spiders, we recommend to study further species, including tests of sublethal parameters, of relative fitness variables, and of possible long-term chronic effects. So far, in field studies about the effect of Bt maize on spiders, web-building spiders were neglected. Considering the high potential exposure of web spiders to Bt maize pollen, more attention should be paid to (web-building) spiders of higher strata in Bt maize risk assessment.

Acknowledgements

We are grateful to Samuel Zschokke for his advice on keeping spiders in the laboratory, and Klemens Ekschmitt for his support. We thank Alfonso Devilli, Hannes Scheuerer, Robert Schrader, Bettina Spindler, Marek Tkaczyk, and Barbara Zeilmaier for field and laboratory assistance and two anonymous reviewers for helpful comments on the manuscript. We thank the Bavarian State Research Center for Agriculture for providing the study fields on the experimental farms. This study was financially supported by the German Federal Ministry of Education and Research (BMBF 0312631 A).

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