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Bt-maize effects on biological parameters of the non-target aphid *Sitobion avenae* (Homoptera: Aphididae) and Cry1Ab toxin detection

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Abstract

Bt-maize crop is increasingly used worldwide and the study of ecological side effects is a major subject in this domain. Under laboratory conditions, we determined Bt-maize effects on the non-target aphid *Sitobion avenae* (Fabricius) (Homoptera: Aphididae). We found no significant differences between *S. avenae* on MON810 and the near-isogenic line when alate offspring production, apterous survivorship, longevity, intrinsic rates of natural increase (r_m), finite rates of increase and doubling times were compared. No significant differences were found between treatments for apterous pre-reproductive and reproductive periods. Additionally, we used immunological tests (ELISA) to detect Cry1Ab protein in maize leaves and *S. avenae* nymphs. Results showed that Bt-maize leaves expressed 0.203 (±0.05) μg Cry1Ab/g leaf tissue (Mean ± SEM). No Cry1Ab protein was present in *S. avenae* nymphs developing on Bt or conventional maize. We conclude that Bt-maize does not affect the development of the non-target aphid *S. avenae* and that Cry1Ab toxin quantities in these aphids are nil, suggesting an inconsequential risk for natural enemies of this aphid species.

Keywords: *Sitobion avenae*; ELISA; Bt-maize; Cry1Ab; Non-target insect; Risk assessment

1. Introduction

Genetically modified (GM) crops are becoming an increasingly important feature of agricultural landscapes. A total of 102 million ha of GM crops were planted worldwide in 2006 with GM-maize being one of the most widely grown GM crops [1]. Among GM-maize varieties, Bt-maize MON810, Bt 11 and event 176 have been genetically modified to express the Cry1Ab toxin [2] which is naturally produced by the bacterium *Bacillus thuringiensis* during its sporulation phase [3]. The Cry1Ab toxin presents insecticidal action against the European Corn Borer (ECB) *Ostrinia nubilalis* (H.) (Lepidoptera: Pyralidae) [4]. In susceptible insects, Cry1Ab crystal proteins produce lesions in the midgut epithelium [5] inducing septicemia caused by enteric bacteria of the exposed insects [6].

Although Bt-toxins are generally considered as highly specific, biological modifications on non-target insects have been reported as a result of exposure to Bt-cultivars [7,8]. However, few studies have been developed to assess risk for non-target phytophagous insects. To date, the non-target phytophagous species most thoroughly studied is the monarch butterfly *Danaus plexippus* L. (Lepidoptera: Danaidae) [9–15]. Aphids are one of the most common phytophagous insects found on maize worldwide [16]. Moreover, they are important prey for many natural enemies, which can be negatively affected when feeding on

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toxic-contaminated prey [16] or prey products [17]. For these reasons, aphids are good tools for studying the effects of Bt crops on non-target phytophagous insects.

One way to estimate the potential risk of Bt crops on non-target phytophagous insects is by estimating the level of potential exposure to Bt-toxin in crops. Immunological analyses can be performed to assess the presence of Bt-toxins on exposed insects and plant products. For Bt-maize, quantification of the Cry1Ab toxin in two non-target aphid species, *Rhopalosiphum maidis* (Fitch) and *R. padi* (L.), has shown that no toxin or only traces of the toxin can be found in Bt-maize-exposed aphids [18–20]. The principal reason for this is the absence of Cry1Ab protein translocation into the phloem of Bt-maize [19]; although traces of Cry1Ab protein on aphids have been explained as a result of intracellular plant sap ingestion during puncture probing [19].

On the other hand, effects on aphids can be related with plant modifications other than the Cry1Ab protein expression, such as pleiotropic effects. It is known, for example, that the content of lignine can be higher in some tissues of Bt-maize [21,22] and this could affect attractiveness to aphids because lignine influences water permeability and the strength of particular cell walls [23]. In this way, the impact may be negative (direct action of the toxin), positive (reduction of competition with other insects), or both (changes in nutritional or physiological quality due to pleiotropic effects of transgene expression). For this reason a complete risk assessment of Bt-crops for non-target aphids also requires the measurements of effects on biological parameters which could be affected by modifications of plant characteristics. Effects of Bt-maize on biological parameters have only been assessed on the aphid *R. padi* [20,24,25]. For this species no effects were found in the first approaches [20,24], although some effects were reported in a posterior study according to the alate or apterous condition of the aphids [25].

Additional to *R. padi*, other aphid species can colonize maize such as *Sitobion avenae* (Fabricius) and *Metopolophium dirhodum* (Walker) [26,27]. *Sitobion avenae* is a major pest of cereals on Europe [28] displaying all life cycle forms known for aphids [29,30]. Generally, it remains on the same host [31,32] and its dispersal induction depends on crowding and food quality [33].

In this context, studies considering these species are warranted because they will contribute to the knowledge of Bt-maize effects on aphid community. For the aphid *R. padi* available information is not consistent about effects of Bt-maize on biological parameters [20,24,25] which indicate that these effects cannot be completely excluded. Studying the effects on more than one aphid species is important in terms of population dynamics, because modifications on biological traits of some species, and thus, on interspecific relationships could result in population dynamic variations [34,35], which justify the study of additional relevant species in the system.

In the present study, we aimed to assess the effects of Bt-maize plants regarding survivorship, demographic parameters and developmental duration periods on the non-target aphid *S. avenae* (F.) (Homoptera: Aphididae). This species can be present in maize crops and cause direct damage and transmit viruses (mainly maize dwarf mosaic virus, MDMV) [26]. We also quantified levels of Cry1Ab toxin in aphids using immunological tests (ELISA) to estimate direct exposure to Cy1Ab protein.

2. Materials and methods

2.1. Biological materials

Standard maize seedlings (AW956 Dekalb Monsanto) were used to rear the host aphid colony of *S. avenae*. For the Bt-maize treatment, the variety 'Novelis', event MON810 (expressing Cry1Ab toxin) was used. For the non-Bt-maize treatment the variety 'Nobilis', a conventional cultivar (Isogenic variety of event MON810), was used. Experiments were carried out when seedlings had 5–6 leaves. Maize was grown in a climatized room at $T = 23 \pm 2$ °C, RH $= 40 \pm 10\%$ with a 16:8 LD photoperiod. Seeds were placed two by two in individual pots ($10 \times 9 \times 8.5$ cm) containing potting mix and watered twice a week with a fertilizer solution.

The *S. avenae* colony was reared using maize seedlings (5–6 leaves) in a room under controlled conditions $T = 23 \pm 2$ °C, RH $= 40 \pm 10\%$ and 16:8 LD. The original strain (c85) was provided by the "Biologie des Organismes et des Populations Appliquée à la Protection des Plantes" (INRA, Rennes, France).

2.2. Effects of Bt-maize on Sitobion avenae development

Alate aphids from the mass reared colony were placed in groups of three in a circular arena (height 0.8 cm; diameter 1 cm) clipped on one maize leaf. Clip cages were randomly assigned to different maize plants and leaves, with a total of 40 clip cages per treatment (i.e. Bt and conventional maize). After 48 h alate aphids were removed and newly laid nymphs were left for 6 days allowing them to establish themselves on plants. After this period of time, each apterous aphid was individually isolated in a clip cage and observed every 2 days. Individuals which became alate were not considered for observation (4.46% and 8.08% of the total offspring for Non-Bt and Bt treatments, respectively).

Apterous aphids were checked to determine survival, pre-reproductive period (i.e. the period of time from birth until the beginning of their reproduction period) and longevity. For each treatment, the offspring were counted and removed on each observation day. Recorded values were divided by the number of living adults to estimate daily fecundity. For each treatment, the intrinsic rate of natural increase r_m was calculated according to the Lotka equation [36] $\Sigma e^{-r_m \chi} l_\chi m_\chi = 1$, where χ is the age, l_χ the age-specific survival and m_χ the age-specific fecundity.

The finite rate of increase $\lambda = e^{r_m}$ and the doubling time ($DT = \ln 2/r_m$) were evaluated according to DeLoach [37].

2.3. Cry1Ab detection and quantification

Presence and quantification of Cry1Ab in different samples were determined using an ELISA kit (EnviroLogix Quantiplate® Kit, Portland, ME, USA). Samples were added to test wells coated with antibodies raised against the Cry1Ab toxin. Cry1Ab present in the sample extracts bound to the antibodies was then detected by the addition of the enzyme (horseradish peroxidase)-labeled Cry1Ab antibody. Results of the assay were visualized with a color development step. Each sample colour was spectrophotometrically measured (at 450 nm) thus obtaining an Optical Density (OD) for each sample. Three calibrators (known Cry1Ab concentrations: 0.5, 2.5 and 5 ppb) were used to establish a linear curve which was used to calculate the Cry1Ab concentration in each sample using its OD. Quantification of sample concentration is only possible if the OD of the sample falls within the range of ODs of the calibrators. The limit of detection of this test was 0.14 ppb Cry1Ab in maize leaf extract. The lowest limit of quantification (ILQ) of Cry1Ab protein was 0.5 ppb of Cry1Ab on the buffer extract which corresponds to the lowest Cry1Ab protein calibrator.

2.4. Bt-maize, conventional leaves and Sitobion avenae analysis

Maize leaf samples were obtained by clamping the leaf with the Eppendorf® tube (2 ml) and its lid, obtaining a leaf sample of 0.5–1 cm^2 on surface. The leaf samples were weighed individually to the nearest 0.01 mg and stored at −20 °C until analysis. A total of 28 samples of leaves per treatment were analysed using the procedure described above.

Aphids (20–30 per clip cage) of mixed stages were placed in rectangular clip cages (length: 3.5 cm; weight: 2.5 cm; height: 0.5 cm) on the lower side of the leaf and allowed to feed during 5 days on Bt and conventional maize. After this period all live aphids for both treatments were recovered and placed in Eppendorf® tubes (20–25 aphids per tube). Each group of aphids was weighed and then stored at −20 °C until analysis. A total of 40–60 aphids per treatment were analyzed.

2.5. Statistical analysis

Survival distribution for each treatment was estimated using the Survival Analysis of Systat® [38] which compared them via a log-rank test using the Mantel–Haenzel method [39]. The effects of Bt-maize on the aphid's developmental periods, demographic parameters, longevities and mean number of descendants per aphid were analyzed using a Wilcoxon signed-rank test [40]. The proportions of alate offspring on Non-Bt and Bt-maize were compared using a chi-square test with Yates' continuity correction. The acceptance level of statistical significance was $P < 0.05$. All analyses were performed using Systat® software [38].

3. Results

3.1. Effects of Bt-maize on Sitobion avenae development

No significant effect of Bt-maize treatment on *S. avenae* survival was observed when compared with conventional maize seedlings ($\chi^2_{MH} = 2.537$; 1 df; $P = 0.111$) (Fig. 1). As for demographic parameters of *S. avenae,* we observed that neither the intrinsic rate of natural increase (r_m), nor the finite rate of increase, nor the doubling time between Bt and conventional treatments was significantly different ($P = 0.795$, 0.758 and 0.272, respectively) (Table 1).

Moreover, there was no significant difference between conventional and Bt-maize regarding the pre-reproductive period ($Z = 1.054$; 1 df; $P = 0.292$), the reproductive period ($Z = 1.424$; 1 df; $P = 0.154$) and the longevity ($Z = 1.651$; 1 df; $P = 0.099$) (Table 1). This was also the case for the number of descendants, equal to 6.0 (±0.9) and 6.1 (±1.2) mean number of nymphs (±SEM) per individual aphid on conventional and Bt treatments, respectively ($Z = 0.427$; 1 df; $P = 0.699$). Similarly, the percentages of alate production on Non-Bt (4.46%) and Bt (8.08%) maize were not significantly different ($\chi^2 = 0.803$; 1 df; $P = 0.370$).

3.2. Cry1Ab detection and quantification

When conventional maize leaves and *S. avenae* aphids fed on conventional and Bt-maize were analyzed, no Cry1Ab protein was detected (Table 2). In contrast, our results showed that Bt-maize leaves contained about 0.203 (±0.05) μg Cry1Ab/g tissue (Table 2).

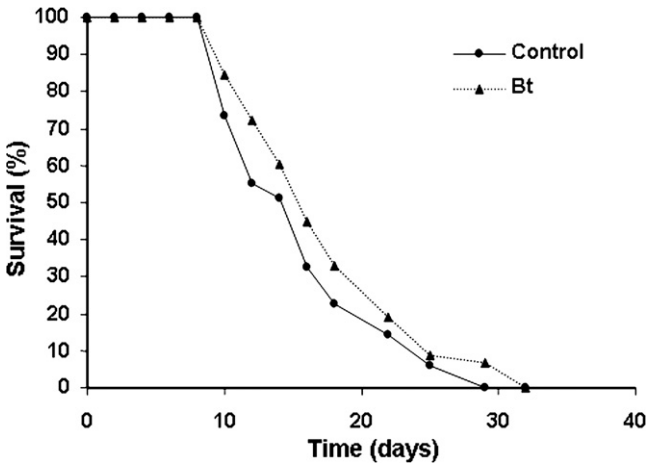

Fig. 1. Survival curves for *Sitobion avenae* F. developing on Bt or conventional maize plants. No significant effect on survival was observed ($\chi^2_{MH} = 2.537$; 1 df; $P = 0.111$).

Table 1
Demographic parameters and developmental periods of the aphid *Sitobion avenae* F. reared on Bt and conventional maize plants

Parameter/period	Conventional ($n = 22$)	Bt ($n = 27$)	Z	P
r_m (female/female/day)	0.11 (±0.02)	0.11 (±0.01)	0.260	0.795
λ (female/female/day)	1.12 (±0.02)	1.12 (±0.02)	0.308	0.758
Doubling time (days)	6.0 (±0.7)	6.5 (±0.8)	1.099	0.272
Pre-reproductive (days)	9.6 (±0.7)	10.8 (±0.7)	1.054	0.292
Reproductive (days)	5.6 (±0.9)	6.4 (±0.8)	1.424	0.154
Longevity (days)	15.2 (±1.1)	17.2 (±1.0)	1.651	0.099

Results are expressed as means (±SEM). r_m, intrinsic rate of a natural increase; λ, finite rate of of increase; n, number of observed aphids; Z, Wilcoxon signed-rank test value; P, P value of the Wilcoxon's test.

Table 2
ELISA analysis for different samples after exposure to Bt or conventional maize plants

Analyzed sample	Mean sample quantity in mg (±SEM)	n	µg Cry1Ab/g tissue (±SEM)
Leaves			
Conventional	14.43 (±1.33)	28	0.0
Bt	14.26 (±1.20)	28	0.203 (±0.05)
S. avena			
Conventional	16.77 (±2.13)	3	0.0
Bt	20.83 (±0.98)	3	0.0

Results are expressed as means (±SEM).

4. Discussion

In the present study no effects on survival, demographic parameters and developmental periods were observed when apterous *S. avenae* were reared on Bt-maize. No Cry1Ab toxin was detected on aphids fed on Bt-maize which suggests that these aphids did not access the toxin during their feeding, which agrees with the absence of effects on their biological parameters. Finally, analyses of Bt-maize leaves confirmed the presence of Cry1Ab toxin in Bt-maize leaves (0.203 µg Cry1Ab/g fresh tissue).

4.1. Effects of Bt-maize on Sitobion avenae development

Our results showing no effect of the Bt-maize expressing the Cry1Ab toxin on *S. avenae* are in concordance with previous studies reporting no impact on the intrinsic rate of increase (r_m) of the non-target phytophagous aphid *R. padi* [20], as with results on developmental time, fecundity and survival of this aphid species [24]. However, Lumbierres et al. [25] observed some effects of Bt-maize on *R. padi*, depending on the aphid form. They reported shorter developmental time of alate individuals reared on Bt-maize, but longer developmental time and lower survival of apterous individuals developed on Bt-maize plants. In a field study, Pons et al. [41] found a higher *S. avenae* density on Bt-maize relative to conventional maize when the Event 176 was tested. These reports compared with our results tend to show that aphid populational and biological responses to Bt crops depend on the aphid species considered as well as the Bt event studied. However, on the basis of the current evidence and the present study, no negative effects from Bt-maize crops are expected for *S. avenae*.

We observed a low reproduction rate of *S. avenae* in our experimental conditions. Assin and Pons [42] found a similar pre-reproductive time, but a longer reproductive period, longevity and higher r_m. As they worked in similar laboratory conditions, differences may have come from the aphid clone or the maize variety. Assin and Pons [42] used a maize variety with a low content of DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoaxin-3-one) which is considered as a common maize defense against aphids [43]. Possibly a higher DIMBOA content in our plants might have restricted development and reproduction of *S. avenae*, but maize varieties with low DIMBOA content are less commonly grown so our data may correspond to realistic *S. avenae* growth on young conventional and Bt-maize.

4.2. Cry1Ab detection and quantification

When Cry1Ab toxin levels were assessed, our results showed that Bt-maize leaves contained about 0.203 µg Cry1Ab/g tissue, which is about 3–32 times lower than those reported previously in studies carried out on the same event (MON810) [44–46]. Variability in expression of Cry1Ab in MON810 has already been reported previously by Nguyen and Jehle [46] who found significant differences of Cry1Ab levels among various plant tissues and developmental stages. This variability seems to occur also with other events such as the event Bt11; Cry1Ab toxin levels found by Raps et al. [19] were 2–7 times higher than those reported by Lynch et al. [47]. Differences in Cry1Ab toxin levels may be related with the tissue, the stage and the plant individuals (including physiological conditions of plants used to conduct the study). However, plant tissue and plant development have been proposed as the main parameters affecting the Cry1Ab contents of transgenic MON810 [46]. In our study, the low quantities of Cry1Ab toxin detected may be related to the analyzed plants. Indeed, the plants used were sown under laboratory conditions (i.e. artificial light and relatively small plastic pots) and therefore were smaller than plants sowed in greenhouse or field conditions.

No quantifiable Cry1Ab toxin level was detected in *S. avenae* fed with Bt-maize. This agrees with previous analy-

ses reporting absence of this toxin in *R. padi* [19] and *R. maidis* fed Bt-maize plants [18]. These results suggest that *S. avenae* has no access to the toxin, which is consistent considering that the Cry1Ab protein is not expressed in Bt-maize phloem [19].

As no Cry1Ab protein was detected on aphids, the lack of effects in their biology seems coherent. However, other effects produced by pleiotropic modifications on plants, such as lignine content [21,22], may be possible. In the present study the lack of effects on biology traits of the aphids exposed to Bt plants indicates that such effects are not expected. This also suggests some substantial equivalence between the Novelis (Bt) and Nobilis (Non-Bt) varieties for the biological parameters studied here, but other traits such as attractiveness onto aphids [48] and also potential variability in plant–aphid interactions [49] would have to be assessed to ensure that the two varieties are equivalent for *S. avenae*.

In terms of risk for natural enemies, Glare et al. [50] distinguished three ways in which Bt-crops may impact on natural enemies: the direct route (direct ingestion), the prey-mediated route (behavioral and/or physiological host–prey changes) and the population level (reduction of host–prey population). In a more detailed analysis, Romeis et al. [51] distinguished (within the direct and the host-mediated route) more specific ways in which natural enemies can be affected. Taking account of these perspectives, the present study suggests no potential risk for *S. avenae* via direct exposure to the Cry1Ab toxin, and also no risk for related natural enemies either via development into the host (parasitoids) or the consumption (predators) of aphids that fed on Bt-maize. Immunological analyses for the detection of Cry1Ab toxin on aphids showed no presence of the toxin which suggested no potential direct exposure to the toxin for *S. avenae* and related natural enemies. No effects on the aphid or natural enemies are expected via the prey-mediated route because aphid development and demographical parameters were not affected under laboratory conditions; however, field confirmation is needed especially since studies in field conditions have revealed from positive to negative effects on other non-target insects [41,52,53].

On the basis of our results we conclude that the Bt-maize expressing Cry1Ab toxin does not affect the development of the non-target phytophagous aphid *S. avenae* on young maize and that no presence of the toxin is detected in this aphid species. This suggests that there is no direct or mediated risk effect at the third trophic level (parasitoids and predators) associated with the aphid *S. avenae* on Bt-maize.

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