Elevated atmospheric ozone increases concentration of insecticidal *Bacillus thuringiensis* (Bt) Cry1Ac protein in Bt *Brassica napus* and reduces feeding of a Bt target herbivore on the non-transgenic parent

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Elevated atmospheric ozone can induce fluctuations in insecticidal protein concentrations in transgenic plants.

ARTICLE INFO

ABSTRACT

Sustained cultivation of *Bacillus thuringiensis* (Bt) transgenic crops requires stable transgene expression under variable abiotic conditions. We studied the interactions of Bt toxin production and chronic ozone exposure in Bt *cry1Ac*-transgenic oilseed rape and found that the insect resistance trait is robust under ozone elevations. Bt Cry1Ac concentrations were higher in the leaves of Bt oilseed rape grown under elevated ozone compared to control treatment, measured either per leaf fresh weight or per total soluble protein of leaves. The mean relative growth rate of a Bt target herbivore, *Plutella xylostella* L. larvae was negative on Bt plants in all ozone treatments. On the non-transgenic plants, larval feeding damage was reduced under elevated ozone. Our results indicate the need for monitoring fluctuations in Bt toxin concentrations to reveal the potential of ozone exposure for altering dosing of Bt proteins to target and non-target herbivores in field environments experiencing increasing ozone pollution.

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1. Introduction

Transgenic crop plants expressing *Bacillus thuringiensis* (Bt) crystal endotoxins (Cry) have been used to control agriculturally important insect pests since their first release in 1996 (James, 2006). Specific Bt toxins limit target herbivore damage to the crop and their use has environmental and human health advantages, such as reduced use of more harmful pesticides (Romeis et al., 2006). Bt toxin resistance evolution in insect herbivores has been raised as a severe threat for the continuing success of Bt-transgenic crops (Tabashnik et al., 2008). Indeed, the frequency of resistance alleles in *Helicoverpa zea* to Bt Cry1Ac cotton have been reported to increase in field populations, but resistance management tactics have been successful in delaying the onset of resistance (Tabashnik et al., 2008).

One factor affecting the possibility of risk of Bt resistance evolution is the fluctuations of Bt toxin concentrations present in the transgenic crops. Variability in Bt toxin concentration occurs due to various factors such as leaf age (Wei et al., 2005; Le et al., 2007), growth condition (Sachs et al., 1998; Le et al., 2007), nutrient availability (Coviella et al., 2002) and CO_2 level (Coviella et al., 2002; Chen et al., 2005; Wu et al., 2007). With regard to continuing the safe use of Bt crops, the study of critical upper and lower limits and possible alterations in Bt toxin concentrations from environmental effects should be considered. In particular, as climate change increases its role for agriculture in the future (IPCC, 2007), interactions of Bt toxin production and abiotic factors such as elevated CO_2, temperature and tropospheric ozone (O_3) should be particularly important to study to determine whether transgenic crop plants will continue to be effective. Elevated background concentrations of tropospheric ozone (O_3) have the potential to affect agricultural plant composition, resource allocation and growth patterns (Ashmore, 2005; IPCC, 2007). O_3 causes variable defence reactions in plants by oxidative stress and leads to severe crop losses in sensitive plants (Ashmore, 2005; Fiscus et al., 2005). Stable transgene expression (i.e. production of Bt toxin), together with endogenous crop fitness is desirable under these future climate conditions. Yet, evidence exists that the concentration of Bt Cry1Ac toxin in transgenic cotton has been reduced by elevated CO_2 mostly through changes in altered nitrogen status (Coviella et al., 2002;

Chen et al., 2005). To our knowledge, the effects of O_3 exposure on Bt toxin concentrations in Bt-transgenic plants are unknown even though tropospheric background concentrations of O_3 and the occurrence of high O_3 episodes have increased because of anthropogenic activities (IPCC, 2007; Sitch et al., 2007). In addition, elevating temperature and CO_2 levels will substantially increase the frequency of tropospheric O_3 episodes by the end of this century (Zeng et al., 2008).

High Bt endotoxin expression is needed to deliver a high dose to target herbivores, which is considered as necessary to delay the evolution of resistance (Bates et al., 2005). There are, however, upper limits of endotoxin synthesis. Another risk for consideration is non-target effects of Bt toxin on beneficial insects, which has been extensively studied in the laboratory, with most of the work using worst-case scenarios, i.e. high doses of Cry toxins to assess potential negative effects on selected organisms present in the agroecosystems (Romeis et al., 2008). It is unlikely that these high doses are ever found in Bt plants in the field, but it is important to bear in mind that Bt toxin can be metabolised or transferred to higher trophic levels (Schuler et al., 1999; Groot and Dicke, 2002; Wei et al., 2008), so the possible effects of changes in Bt toxin concentrations in crop plants could have consequences affecting, in addition, insects of higher trophic levels.

Insect feeding pattern is another ecologically important factor related to insect control by transgenic plants that should be considered in future climates, since transgenic Bt plants require target herbivore feeding for Bt toxin uptake. Herbivore feeding patterns and preferences might be altered in future climatic conditions because of, e.g. allocation changes in response to oxidative stress and changes in total protein and total amino acid levels and the nutrient metabolism of plants (Trumble et al., 1987; Holton et al., 2003; Fiscus et al., 2005; Valkama et al., 2007), or direct effects of O_3 on insect physiology or behaviour (Awmack et al., 2004), e.g. attraction towards the host plant. As a continuum, these kind of changes might affect the ecology of both target and non-target herbivore insect species and alter Bt toxin uptake and dosing. Chen et al. (2005) found both Bt transformation and elevated CO_2 to negatively affect the growth parameters of Helicoverpa armigera in cotton, but to our knowledge, the only study to assess O_3 exposure effects on herbivory in Bt-transgenic plants is our previous work, where we found no effect by chronic 75 or 150 ppb O_3 elevation on short-term mean relative growth rate of Plutella xylostella L. larvae on Bt oilseed rape (Himanen et al., 2008a).

Here, we describe the use of Bt Cry1Ac-transgenic Brassica napus ssp. oleifera, oilseed rape (Halfhill et al., 2001), as a model Bt plant for ozone–herbivory interaction studies. Bt Cry1Ac enables the control of lepidopteran larvae, including the cosmopolitan Brassica pest P. xylostella L. (diamondback moth). P. xylostella has evolved resistance to Bt toxin in laboratory studies and in the field (Tabashnik et al., 2003). Therefore, it is essential to evaluate Bt toxin concentration changes in Bt oilseed rape since one of its target pests has a high potential to evolve resistance. Furthermore, low toxin content could hasten resistance. We have sufficient background knowledge about Bt toxin concentrations in this model crop when grown in growth chambers, greenhouse and the field (e.g. Halfhill et al., 2001; Zhu et al., 2004; Wei et al., 2005; Le et al., 2007; Himanen et al., 2008b), which facilitates comparison of effects caused by environmental factors, such as O_3 here, on the variation caused by, e.g. growth condition. The goal of this study was to predict whether the transgenic defence trait (Bt toxin production) will be effective under elevated atmospheric O_3 concentrations in Bt oilseed rape, and to assay effects of O_3 exposure on target herbivore feeding patterns and their performance on sensitive (non-transgenic) and resistant (Bt-transgenic line) oilseed rape.

2. Materials and methods

Oilseed rape (B. napus ssp. oleifera L.) cv. Westar transformed to contain a truncated synthetic Bt cry1Ac transgene (courtesy of Mycogen) and a GFP (green fluorescent protein) mgfp5-er (courtesy of Jim Haseloff) marker gene (Halfhill et al., 2001) event 'GT1' was selected for use in these experiments. It is a very well-characterized Bt oilseed rape line, which contains a single insert and has moderate level of transgene expression with no apparent fitness costs (Halfhill et al., 2001). Equal numbers of non-transgenic cv. Westar parent line and transgenic Bt Cry1Ac F_4 seeds were sown in 0.66 l pots in 2:1:1 fertilized compost (Kekkilä, Finland, N–P–K: 100–30–200 mg l^{-1}):B2 Sphagnum peat (Kekkilä, Finland, N–P–K: 110–40–220 mg l^{-1}):sand mixture and grown together in four identical computer-controlled growth chambers (2.6 m^3, Bioklim 2600T, Kryo-Service Oy, Helsinki, Finland) under 16 L: 8 D photoperiod (light adjusted to PAR of approximately 250 μmol m^{-2} s^{-2}) and 20/16 °C temperature. The plants were watered daily. Four chambers were available for use simultaneously and these were each set to one of the four O_3 regimes; filtered air control, 50, 75 or 100 nl l^{-1} (ppb). Chronic O_3 exposure was run for 8 h daily, from 8:30 am to 4:30 pm (from the 3rd day after sowing). O_3 was generated from pure oxygen with an O_3 generator (Fisher OZ 500 Ozone generator, Bonn, Germany), and continuously monitored with an O_3 analyzer (Environnement S.A., Model O_3 42M, Poissy, France). Two repetitions of the experiment were conducted in time to control for chamber effect by using replication as a random factor in the statistical analysis, where individual plants served as replicates. Also, to avoid any effects of chamber-specific growth conditions, the treatments were rotated weekly between the four similar chambers used, and the plants were rotated inside the chambers at the same time.

Bt Cry1Ac-susceptible P. xylostella L. (Lepidoptera: Yponomeutidae), diamondback moth (DBM), larvae originating from a colony reared on broccoli (Brassica oleracea ssp. italica) at 12 L:12 D, approximately 25 °C and 50% relative humidity at the University of Kuopio were used in the target herbivore growth assessments. P. xylostella is a Brassica specialist and a cosmopolitan pest (Talekar and Shelton, 1993). For determining P. xylostella mean relative growth rate (MRGR), seven 21-d old non-Bt and Bt plants from control and each O_3 treatment were randomly selected. The 1st true leaf from five of these plants per treatment was collected for Bt toxin analysis, immediately deep-frozen in liquid nitrogen, and stored at −80 °C for total soluble protein and Cry1Ac protein analysis. The 3rd true leaf was detached from each plant, and the petiole was put into a 2.0 ml eppendorf tube filled with tap water, and sealed with parafilm. Thereafter, the leaves were placed into 200 ml plastic containers (Nalgene) and the lids were replaced with fine mesh to allow evaporation. Four 2nd instar DBM larvae were group-weighed, and placed on each leaf. Bt and non-Bt leaves were placed in the same growth chambers, with the O_3 treatment conditions described above. After 48 h, the larvae were collected, their possible mortality recorded, and living larvae were group-weighed. The leaves were photographed to determine the leaf area eaten by the larvae in pixels, which were converted to square centimeters (Adobe Photoshop Elements 2.0). We also calculated the percentage of damaged area of the total leaf area, since the treatments could also affect the size and shape of the leaves. MRGR of the DBM larvae was calculated with the equation: [ln (final weight of larvae) − ln (initial weight of larvae)]/duration of feeding in days (Van Emden, 1969).

The amount of total soluble protein (TSP) was measured with the Bradford (1976) method and Cry1Ac concentration with a commercial enzyme-linked immunosorbent assay (ELISA) PathoScreen kit for Cry1Ac (Agdia, Elkhart, Indiana, US) as in Vojtech et al. (2005), except that each sample was tested in duplicate and approximately 10 μg of total protein was added to sample wells. The optical density (OD) of each sample was read at 620 nm wavelength with an ELISA plate reader (Easy Reader SF Plus, SLT Labinstruments).

Before the statistical analysis the normality and the equality of error variances of variable residuals were tested and some variables were log $(x + 1)$ or square-root transformed for normality. Linear mixed model was used for assessing main effects of plant type and O_3 level (fixed factors) and their interaction effects on Bt Cry1Ac concentration, total soluble protein, DBM mean relative growth rate and DBM feeding area. The model included repetition of the experiment as a random factor in all analyses and initial larval weight as a covariate in MRGR and feeding area analysis. Mixed model post hoc tests based on estimated marginal means with Bonferroni correction were used for comparing treatments within plant type.

3. Results

There was significantly increased Bt Cry1Ac toxin protein concentration in transgenic oilseed rape leaves in the 100 ppb elevated O_3 treatment, measured either per leaf fresh weight or per total soluble protein of leaves (mixed model, main effect of O_3: $F_{3,35} = 6.889$, $P = 0.001$ and $F_{3,35} = 8.331$, $P < 0.001$, respectively) (Table 1). Ozone had no statistically significant effect on the amount of total soluble protein in the leaves (mixed model, main effect of O_3: $F_{3,35} = 2.457$, $P = 0.079$) (Table 1).

Table 1
Mean ± SEM Bt Cry1Ac (Bt toxin) concentrations as µg g^{-1} leaf FW (fresh weight) and ng mg^{-1} total soluble protein (TSP), and the amount of TSP (mg g^{-1} leaf FW) in Bt-transgenic *B. napus* plants grown under filtered air (control), 50, 75 or 100 ppb of elevated O$_3$ (8 h daily)

Treatment	µg Cry1Ac (g^{-1} FW)	ng Cry1Ac (mg^{-1} TSP)	TSP (mg g^{-1} FW)
Control	1.352 ± 0.163[a]	255.6 ± 40.7[a]	5.792 ± 0.364[a]
50 ppb O$_3$	1.227 ± 0.189[a]	225.1 ± 27.5[a]	5.658 ± 0.387[a]
75 ppb O$_3$	1.833 ± 0.234[ab]	334.7 ± 36.9[ab]	5.670 ± 0.243[a]
100 ppb O$_3$	2.323 ± 0.166[b]	476.1 ± 45.0[b]	5.124 ± 0.242[a]

Statistically significant differences ($P < 0.05$) between treatments are marked with different letters (linear mixed model post hoc tests based on estimated marginal means), $n = 10$.

Mean relative growth rate of *P. xylostella* larvae feeding on Bt oilseed rape was negative in all O$_3$ treatments and therefore reduced compared to that of larvae feeding on the non-transgenic plants (mixed model, main effect of plant type: $F_{1,103} = 811.7$, $P < 0.001$). Elevated O$_3$ had no statistically significant effect on MRGR of *P. xylostella* on non-transgenic or Bt-transgenic plants (Fig. 1).

Less leaf area was consumed from the non-transgenic plants by the *P. xylostella* larvae under 100 ppb (expressed as cm^2 and % of total leaf area, Fig. 2a and b) and under 75 ppb (% of total leaf area, Fig. 2b) elevated O$_3$ compared to control treatment (mixed model, main effect of O$_3$: $F_{3,51} = 3.613$, $P = 0.019$ for leaf area fed in cm^2 and $F_{3,51} = 3.587$, $P = 0.020$ for % of total leaf area fed). Elevated O$_3$ did not have this kind of effect on larval feeding area in Bt oilseed rape leaves (mixed model, interaction plant type × O$_3$, $F_{3,103} = 3.489$, $P = 0.018$ for leaf area fed in cm^2 and $F_{3,103} = 3.276$, $P = 0.024$ for % of total leaf area fed), where feeding damage levels were very low in all treatments due to the presence of Bt toxin (mixed model, main effect of plant type, $F_{1,103} = 173.66$, $P < 0.001$ for leaf area fed in cm^2 and $F_{1,103} = 44.137$, $P < 0.001$ for % of total leaf area fed). The total leaf area eaten on Bt plants was very small (range of feeding area 0–0.021 cm^2 per leaf) and, on average, 50 times less leaf area was consumed in Bt than in non-Bt plants.

4. Discussion

Bt Cry1Ac concentrations were higher in Bt oilseed rape leaves under high level of chronic O$_3$ exposure (100 ppb) compared to plant leaves grown under no O$_3$. Previously, the Bt Cry1Ac concentration increased on oilseed rape leaves as they aged (Wei et al., 2005; Le et al., 2007) and correspondingly, the amount of TSP
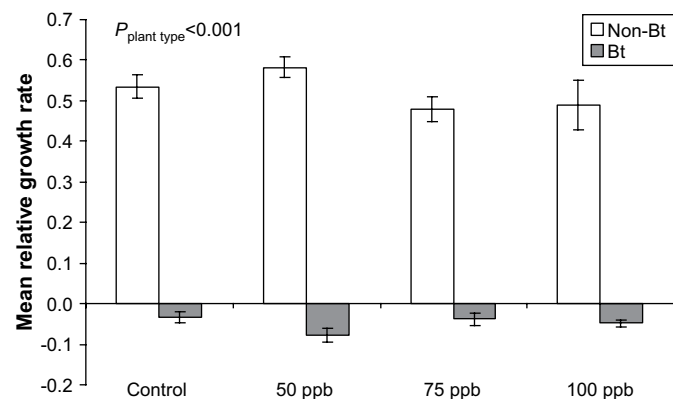
Fig. 1. Mean relative growth rate ± SEM of diamondback moth (*P. xylostella* L.) larvae feeding on non-transgenic (non-Bt) and Bt-transgenic (Bt) *B. napus* plants grown and tested under control (filtered air), 50, 75 or 100 ppb O$_3$ treatment. Statistically significant main effects of plant type and ozone and their interactions are shown (linear mixed model), $n = 14$.
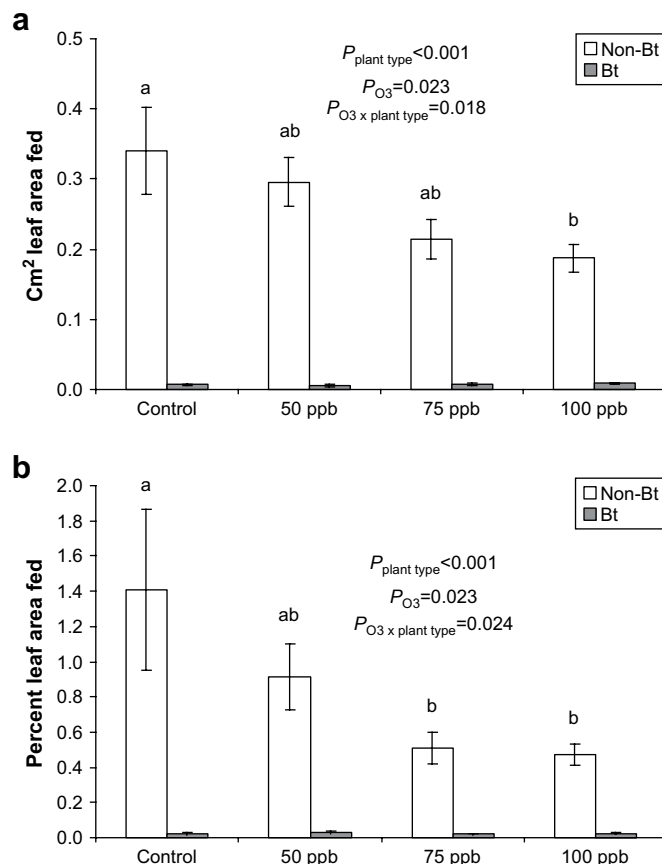
Fig. 2. Leaf area consumed ± SEM by diamondback moth (*P. xylostella* L.) larvae (a) as cm^2 and (b) as percent of total leaf area in non-transgenic (non-Bt) and Bt-transgenic (Bt) *B. napus* plants grown and tested under control (filtered air), 50, 75 or 100 ppb O$_3$ treatment. Statistically significant main effects of plant type and ozone and their interactions are shown (linear mixed model). Different letters indicate statistically significant differences between O$_3$ treatments among plant type (mixed model post hoc tests), $n = 14$.

either decreased (Wei et al., 2005) or showed an increase throughout the vegetative stage followed by a decrease or a static stage during pod formation (Le et al., 2007), which demonstrates that Cry1Ac is a relatively stable protein. Since one typical symptom of chronic O$_3$ stress in plants is premature senescence (Fiscus et al., 2005), this observation suggests that earlier maturation could be one reason for the observed increase in Cry1Ac under these conditions. However, in a previous study with this oilseed rape line, no significant differences in Bt toxin concentrations occurred in plants grown under elevated CO$_2$ and temperature, singly or in combination, although the growth of the plants was highly affected by these abiotic factors (Himanen et al., 2008b). This indicates that elevated O$_3$ acts more strongly than these other climate change factors on Bt toxin concentration of Bt oilseed rape. In this study, Bt toxin production remained robust under O$_3$ elevations and accordingly increased its proportion of TSP with O$_3$ exposure. Interestingly, the increase in Bt toxin concentration was higher with increasing dose of O$_3$ as revealed by the comparison of low (50 ppb) and medium exposures (75 ppb) to high O$_3$ exposure (100 ppb), and the statistical significant difference compared to control treatment appeared only with the highest exposure. The TSP concentration of leaves was not significantly affected by O$_3$ treatment in this study, although some previous studies have reported increases in TSP after O$_3$ exposure because of the release of soluble nitrogenous compounds from plant structures by O$_3$ action (Trumble et al., 1987).

The variation of Bt toxin concentration in the leaves of Bt oilseed rape in this study was in the range found previously with the same transgenic line in growth chamber (Himanen et al., 2008b), greenhouse (Wei et al., 2005) and field conditions (Le et al., 2007), and variations between different field locations (Zhu et al., 2004; Le et al., 2007). However, our results emphasize the importance of O_3 studies in a wider scale, i.e. in field conditions over several years, where other abiotic factors may interact and mediate the O_3 response of Bt toxin as well. This is important for Bt-transgenic crops in general, and in our case, it would be particularly interesting to assay whether the O_3 effect observed here is rather growth condition-specific, i.e. more pronounced in either controlled or natural environment. Since the Bt toxin concentration was shown to increase both per plant fresh weight and per total soluble protein in plants grown in environment-controlled growth chambers, there is a considerable reason to believe that this might occur in field conditions and with other plant species as well. Nevertheless, the O_3 effects on Bt concentration must be placed in context to other environmental factors present in field environments to predict transgene expression stability needed in agriculture, which is also ecologically important. Previous studies have revealed elevated CO_2 as a climate change factor that reduces Bt toxin concentration in Bt cotton plants grown both in controlled and field conditions (Coviella et al., 2002; Chen et al., 2005; Wu et al., 2007), and presumably the difference is also related to nitrogen availability. It is unknown what this effect might mean for Bt cotton in agriculture. As the current climate change will induce rises in both CO_2 and O_3 levels in the troposphere, the combined effects of elevated CO_2 and O_3 on Bt toxin concentrations of Bt plants should also be considered, as their effects might be opposing.

Mean RGR of *P. xylostella* larvae on Bt oilseed rape was negative in all treatments, indicating that the Bt trait is robust under O_3 elevation. A similar result was found previously in a 24 h assessment of *P. xylostella* mean growth rate under chronic 75 and 150 ppb O_3 elevation in this oilseed rape line (Himanen et al., 2008a). The MRGR of *P. xylostella* was not affected by O_3 elevation in the non-transgenic plants here or in our previous study, where there was no difference in the MRGR of *P. xylostella* larvae feeding on non-transgenic Westar parent line under control, 75 or 150 ppb O_3. In this study there was less leaf area eaten from non-transgenic plants in the elevated O_3 treatments, which suggests that O_3 as such rendered the oilseed rape plant less favourable and attractive for the herbivore, and therefore, might actually diminish crop losses caused by *P. xylostella* larvae. However, lower O_3 exposure (50 and 75 nl l^{-1}) did not significantly reduce the larval feeding, and the overall effects of high O_3 (100 nl l^{-1}) on plant fitness (i.e. lowered photosynthetic rate and biomass) (Fiscus et al., 2005; Himanen et al., 2008a) are assumed to be more harmful, and negate any benefits obtained from potential decreased herbivory.

Since the herbivores were fed with leaves from O_3-grown plants and their feeding was assayed under the same O_3 treatment, both indirect (plant-mediated) and direct effects of O_3, as present together in nature, affected herbivore performance and feeding in this study. The indirect (i.e. plant-mediated) effect of O_3 is typically to render leaves more palatable to herbivores leading to higher leaf damage levels and weight gain in insects because of enhanced nutritional value, better digestibility of plant material or alterations in secondary compounds (Trumble et al., 1987; Jones and Coleman, 1988; Bolsinger et al., 1992; Jondrup et al., 2002; Percy et al., 2002). O_3 can induce changes such as rise in TSP as evidenced by Trumble et al. (1987) on tomato (*Lycopersicon esculentum*) plants, but also cause fluctuations in total free amino acid content or specific amino acid composition (Trumble et al., 1987), which can be critical for the development of certain herbivores through direct effects or by changes in secondary compound allocation. However, the O_3 response is somewhat related to the insect or plant species studied,

instar used and O_3 exposure length (Holopainen, 2002; Agrell et al., 2005; Valkama et al., 2007). O_3 can, on the other hand, induce defence responses (Fiscus et al., 2005) and increase concentrations of secondary compounds (Himanen et al., 2008a), some of which might also protect plants from herbivory. For example, altered feeding patterns in *Brassica* plants specifically could be affected by changes in the levels of glucosinolates, the amino acid-derived secondary compounds of *Brassica*s, concentrations of which have been altered by elevated O_3 (150 nl l^{-1}) in Bt and non-Bt oilseed rape (Himanen et al., 2008a). *P. xylostella* has a resistance mechanism against glucosinolates (Ratzka et al., 2002), which suggests, however, that other secondary compound groups, such as proteinase inhibitors present in crucifers in significant amounts, possessing deterrent effects on DBM larvae (De Leo et al., 2001), could be higher in the plants also under oxidative stress. Unfortunately, we did not analyse proteinase inhibitors in this study, but measuring the response of these to elevated O_3 together with an analysis of DBM performance in oilseed rape could reveal whether these two factors are related.

Direct effects of O_3 on herbivores are predicted to have relatively low impact compared with plant-mediated effects at current O_3 levels (Awmack et al., 2004). This view is bolstered by the prevalence of studies reporting mostly positive effects of O_3 elevation on herbivores (Valkama et al., 2007). In addition, e.g. Levy et al. (1974) demonstrated that two cockroach species and a fire ant species were unaffected by prolonged elevated O_3 exposure. Ozone is, however, a cell damaging agent and it can be used to directly kill stored-product insects using high O_3-fumigation doses (typically over 200 ppb is needed, Hollingsworth and Armstrong, 2005). Therefore, the net effect of O_3 exposure on different insect species is still not conclusive. Importantly, the growth (MRGR) of the *P. xylostella* larvae in our study was similar in control and high O_3, so it is possible that the nutritional quality of plant material for larvae was better in high O_3 leaves, but the reduced feeding behaviour caused by deterrent secondary compounds or direct effects of O_3 led to the observed response of no change in the MRGR of the herbivores.

The relation of insect feeding preference and exposure to Bt toxin is important since increased Bt toxin concentrations present in leaves grown under O_3 exposure combined with possible increased feeding by herbivores non-susceptible to Bt toxin, unlike *P. xylostella*, might result in higher doses of toxin transmitted in ecosystems by non-target herbivores to higher trophic levels (Schuler et al., 1999; Groot and Dicke, 2002; Wilkinson et al., 2003; Wei et al., 2008). Wei et al. (2008) have shown that Cry1Ac does travel through trophic levels in amounts high enough to be detected via ELISA.

5. Conclusions

We found that high chronic O_3 increased Bt Cry1Ac toxin concentration in Bt-transgenic oilseed rape leaves but did not change the levels of target herbivore feeding or their performance on Bt plants. The susceptible oilseed rape parent line, on the other hand, experienced reduced feeding damage by *P. xylostella* larvae with high O_3, but the MRGR of the herbivores was unaffected by O_3 suggesting that food quality for the larvae was improved. This study emphasizes the importance to assay Bt toxin concentration fluctuations in response to abiotic factors, of which O_3 exposure is a good example. Field testing is needed to reveal whether environmentally realistic O_3 concentrations in polluted areas lead to similar responses as observed in this study under controlled conditions. Higher ozone might lead to altered dosing of transgenic proteins in target and non-target herbivores and insects of higher trophic levels in two ways: through altered Bt toxin concentrations in plant material and changes in herbivore feeding patterns.

Acknowledgements

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