Environmental Pollution 157 (2009) 181–185

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

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

Sari J. Himanen [a,*], Anne-Marja Nerg [a], Anne Nissinen [a,b], C. Neal Stewart, Jr. [c], Guy M. Poppy [d], Jarmo K. Holopainen [a]

[a] University of Kuopio, Department of Environmental Science, P.O. Box 1627, FIN-70211 Kuopio, Finland
[b] MTT Agrifood Research Finland, Plant Protection, FIN-31600 Jokioinen, Finland
[c] University of Tennessee, Department of Plant Sciences, Knoxville, TN 37996-4561, USA
[d] University of Southampton, School of Biological Sciences, Southampton SO16 7PX, UK

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

ARTICLE INFO

Article history:
Received 27 March 2008
Received in revised form 2 July 2008
Accepted 17 July 2008

Keywords:
Bacillus thuringiensis
Elevated ozone
Herbivory
Plutella xylostella L.
Transgenic plants

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.

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;

* Corresponding author. Tel.: +358 17 163177; fax: +358 17 163191.
E-mail address: sari.himanen@uku.fi (S.J. Himanen).

0269-7491/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.envpol.2008.07.006

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).