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Effects of elevated carbon dioxide and ozone on volatile terpenoid emissions and multitrophic communication of transgenic insecticidal oilseed rape (*Brassica napus*)

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Summary

- Does transgenically incorporated insect resistance affect constitutive and herbivore-inducible terpenoid emissions and multitrophic communication under elevated atmospheric CO_2 or ozone (O_3)? This study aimed to clarify the possible interactions between allocation to direct defences (*Bacillus thuringiensis* (Bt) toxin production) and that to endogenous indirect defences under future climatic conditions.
- Terpenoid emissions were measured from vegetative-stage non-Bt and Bt *Brassica napus* grown in growth chambers under control or doubled CO_2, and control (filtered air) or 100 ppb O_3. The olfactometric orientation of *Cotesia vestalis*, an endoparasitoid of the herbivorous diamondback moth (*Plutella xylostella*), was assessed under the corresponding CO_2 and O_3 concentrations.
- The response of terpenoid emission to CO_2 or O_3 elevations was equivalent for Bt and non-Bt plants, but lower target herbivory reduced herbivore-inducible emissions from Bt plants. Elevated CO_2 increased emissions of most terpenoids, whereas O_3 reduced total terpenoid emissions. *Cotesia vestalis* orientated to host-damaged plants independent of plant type or CO_2 concentration. Under elevated O_3, host-damaged non-Bt plants attracted 75% of the parasitoids, but only 36.8% of parasitoids orientated to host-damaged Bt plants.
- Elevated O_3 has the potential to perturb specialized food-web communication in Bt crops.

Introduction

Climate change and the expanding use of transgenically modified crop cultivars are predicted to be among the most important issues affecting agriculture in the coming decades, and are the subjects of extensive research efforts (James, 2007; IPCC, 2007). Production of *Bacillus thuringiensis* (Bt) crystal endotoxin (Cry) proteins in a crop plant limits specific herbivorous insect attack without the need for chemical treatments (Romeis *et al.*, 2006). However, Bt toxin production requires an incremental resource investment for the plant. The possibility of competition between constitutive Bt toxin production and allocation to secondary metabolism under changed abiotic conditions related to climate change (Coviella *et al.*, 2002; Chen *et al.*, 2005) led us to assay secondary metabolism, in the form of volatile organic compounds (VOCs), in Bt Cry1Ac-transgenic oilseed rape (*Brassica napus*) and its non-transgenic parent line under atmospheric CO_2 or O_3 elevations. We extended this evaluation to investigate the ecological effects that these possible changes in VOC emissions might have through effects on multitrophic communication.

The emission of inducible VOCs by plants in response to herbivore feeding attracts predators and parasitoids, that is,

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insects of higher trophic levels, to prey or host, limiting further herbivore attack (indirect defence) (Dicke *et al.*, 1990; Turlings *et al.*, 1990; Du *et al.*, 1996). Increases in plant VOC emissions are related to both the quantity and the quality of herbivore feeding damage (e.g. De Moraes *et al.*, 1998; Turlings *et al.*, 1998). Bt toxin in transgenic Bt plants inhibits target herbivore feeding and it is reasonable to suppose that reduced feeding damage by Bt-sensitive herbivores can reduce the induction of VOC emissions (Schuler *et al.*, 1999a,b). Differences in VOC emissions from intact Bt cotton (*Gossypium hirsutum*; Yan *et al.*, 2004) and herbivore-damaged Bt maize (*Zea mays*; Turlings *et al.*, 2005; Dean & De Moraes, 2006) and oilseed rape (Ibrahim *et al.*, 2008) have been found, compared with their nontransgenic parent lines. Importantly, Bt plants do allow herbivore feeding initiation and it is therefore possible that this might be adequate for VOC induction. Some quantitative differences in constitutive VOC emissions between transgenic plants and their parent lines are to be expected (Yan *et al.*, 2004), for example as a result of differences in phenotype produced by the introduced trait (Himanen *et al.*, 2008a), whereas possible qualitative differences indicating allocation differences could be more ecologically relevant. With VOC emissions, the genetic variation among plant cultivars can be high, as shown for example in maize lines (Degen *et al.*, 2004). Substantial equivalence analysis of transgenic crop varieties examines how the composition of the transgenic cultivar fits into the scale of variation found among traditionally bred cultivars (Kok & Kuiper, 2003). From an ecological perspective, this kind of comparative analysis of VOC profiles could also be useful in detecting possible risks attributable to alterations in multitrophic interactions (Turlings *et al.*, 2005).

The first aim of this study was to compare the herbivore-induced volatile emission profiles of nontransgenic and Bt-transgenic oilseed rape plants after target herbivory, defining the role of insect resistance for endogenous VOC defence activation in this model species. We also wanted to investigate whether a specialized tritrophic interaction among oilseed rape, the Bt-target herbivore *Plutella xylostella* and its endoparasitoid *Cotesia vestalis* (Hymenoptera: Braconidae) is affected by Bt production. Schuler *et al.* (1999a) showed that the parasitoid *C. vestalis* preferred plants damaged by Bt-resistant larvae over those on which Bt-susceptible larvae were feeding, which provides an indication of the potential of feeding damage to affect multitrophic communication in Bt oilseed rape. However, Turlings *et al.* (2005) demonstrated that the parasitoids *Cotesia marginiventris* and *Microplitis rufiventris* did not distinguish between equally damaged non-Bt and Bt maize despite quantitative VOC differences in their profiles. Potential differences in VOC induction in Bt plants might lead to changes in multitrophic interactions in crop ecosystems and surroundings, although these effects have to be compared with those of conventional pest control practices such as the use of chemical insecticides, which often have more harmful ecological effects (Romeis *et al.*, 2006). Chemical control is in many cases unspecific and directly affects numerous beneficial insect species negatively (Romeis *et al.*, 2006).

The second aim of our study was to determine whether changed abiotic conditions related to future climatic conditions, namely elevated atmospheric CO_2 or O_3 exposure, affect the induction of VOC emissions and tritrophic communication comparably in non-Bt and Bt oilseed rape, with the latter allocating resources to constitutive Bt toxin production. Depending on the plant species and individual compounds, terpenoid emissions have been found to increase, to decrease or to remain unaffected under elevated concentrations of CO_2 (Constable *et al.*, 1999; Loreto *et al.*, 2001; Staudt *et al.*, 2001; Niinemets *et al.*, 2004; Vuorinen *et al.*, 2004a,b, 2005). However, C_5-isoprenoid isoprene emission has been consistently observed to decrease under elevated CO_2 (Rosenstiel *et al.*, 2003). O_3 stress can increase VOC emissions from plants (Heiden *et al.*, 1999; Vuorinen *et al.*, 2004c; Beauchamp *et al.*, 2005; Rinnan *et al.*, 2005), through increased release or synthesis. In addition, isoprene seems to protect plants against oxidative stress by stabilizing cellular membranes (Loreto & Velikova, 2001; Loreto *et al.*, 2001). Also, O_3 interacts with VOCs present in the atmosphere to form secondary aerosol particles (Pinto *et al.*, 2007a), and extinction times are low especially for the most reactive sesquiterpenes (Atkinson & Arey, 2003). The net effect of these complex factors acting together could produce a reduction, an increase, or no net change in VOCs available for parasitoid olfactory detection with O_3 elevation.

Considering the functioning of multitrophic interactions in future atmospheres, O_3 exposure has not been found to disturb the orientation of the specialist predatory mite *Phytoseilus persimilis* on prey-infested *Phaseolus lunatus* or the parasitoid *C. vestalis* on host-damaged *Brassica oleracea* (Vuorinen *et al.*, 2004c; Pinto *et al.*, 2007b, 2008). However, elevated CO_2 exposure resulted in cultivar-specific attractiveness in the orientation behaviour of *C. vestalis* towards host-damaged *B. oleracea* (Vuorinen *et al.*, 2004a), indicating possible changes in tritrophic interactions. To our knowledge, no previous studies of VOC emissions and tritrophic interactions under elevated CO_2 or O_3 have been performed using Bt-transgenic plants. However, these factors could be important when assessing the ecological effects of insect-resistant plants and determining whether Bt crop systems might have lower efficiency in attracting natural enemies under elevated CO_2 or O_3.

We hypothesized, firstly, that Bt oilseed rape plants might have reduced VOC emissions compared with the non-Bt parent line. Constitutive emissions could be reduced by allocation changes as a result of investment in Bt toxin production (Coviella *et al.*, 2002; Chen *et al.*, 2005) and subsequent delayed growth (Himanen *et al.*, 2008a), whereas inducible emissions could be lower as a result of reduced target herbivory (Dean & De Moraes, 2006). Secondly, we expected that, under elevated CO_2, terpenoid emissions would be

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decreased in both plant types because of simultaneous increasing pyruvate competition for higher photosynthesis levels (Rosenstiel *et al.*, 2003) and this would, again, be more pronounced in the Bt-transgenic plants investing in Bt toxin production. Our third hypothesis was that O_3 exposure causing oxidative stress and allocation to synthesis of defence proteins (Fiscus *et al.*, 2005) could lead to increases in VOC emissions (Vuorinen *et al.*, 2004c), but the presence of O_3 in the gas phase would lead to atmospheric degradation reactions and to a net decrease in VOCs detectable to parasitoids (Pinto *et al.*, 2007b). Bt-transgenic plants, having the extra investment cost of Bt toxin protein synthesis, but being subject to lower herbivory, were hypothesized to have lower induction of emissions in response to elevated O_3 compared with non-Bt plants. In addition, the degradation of terpenoids in the gas phase could reduce the VOCs attracting parasitoids even more in the case of Bt-producing plants.

Materials and Methods

Plants, herbivores and parasitoids

Oilseed rape (*Brassica napus* ssp. *oleifera* L.) cv. Westar transformed to contain a truncated synthetic Bt *cry1Ac* transgene (courtesy of Mycogen, Dow AgroSciences, Indianapolis, IN, USA) and a green fluorescent protein (GFP) *mgfp5-er* (courtesy of Jim Haseloff) marker gene, event GT1 (Halfhill *et al.*, 2001), was selected for use in these experiments. This event contains a single homozygous insert and produces effective transgene expression with no apparent fitness costs. F_4 seeds of Bt-transgenic and nontransgenic oilseed rape (cv. Westar parent line) sown in 0.66-l pots in a 2 : 1 : 1 fertilized compost (Kekkilä, Tuusula, Finland; nitrogen:phosphorus:potassium (N:P:K) 100 : 30 : 200 mg l^{-1}): B2 *Sphagnum* peat (Kekkilä; N:P:K 110 : 40 : 220 mg l^{-1}): sand mixture were used in all experiments. Plants were grown in computer-controlled growth chambers (2.6 m^3; Bioklim 2600T; Kryo-Service Oy, Helsinki, Finland) under a 16 : 8 h light:dark photoperiod (light adjusted to photosynthetically active radiation *c.* 250 μmol m^{-2} s^{-1}) with 20 : 16°C day:night temperature and daily watering. CO_2 and O_3 effects were studied in separate experiments.

The herbivores used were Bt Cry1Ac-susceptible diamondback moth (DBM; *Plutella xylostella* L. (Lepidoptera: Yponomeutidae)) larvae originating from a colony reared on broccoli (*Brassica oleracea* ssp. *italica*) under a 12 : 12 h light:dark photoperiod, *c.* 25°C and 50% relative humidity at the University of Kuopio. *Plutella xylostella* is a cosmopolitan pest and a specialist on *Brassica* species (Talekar & Shelton, 1993). As parasitoids we used *Cotesia vestalis* (Haliday) (Hymenoptera: Braconidae) (previously known as *Cotesia plutellae* (Kurdjumov)), a specialist solitary endoparasitoid preferably attacking *P. xylostella* early instar larvae. *Cotesia vestalis* parasitoids were reared on DBM larvae (second instar larvae offered for egg laying) on broccoli at the University of Kuopio. Emerging *C. vestalis* parasitoids were collected daily into plastic insect cages, of which two sides were covered with nylon mesh to allow ventilation. They were fed on water-honey solution, and 1- to 3-d-old females were used in the Y-tube assay.

Expt 1: VOCs from CO_2 treatments and tritrophic interactions

Equal numbers of nontransgenic and Bt-transgenic plants were sown in two growth chambers (one at 360 μl l^{-1} and one at 720 μl l^{-1} CO_2, supplied from gas tanks, 24 h daily, with concentrations monitored continuously, as in Vuorinen *et al.*, 2004a). The experiment was repeated twice because of the limited number of growth chambers available simultaneously. The repetition was used as a blocking factor in the statistical analysis to avoid pseudoreplication issues, as individual plants served as replicates. Also, to avoid any effects of chamber-specific growth conditions, the treatments were rotated weekly between the two similar chambers used, and the plants were rotated inside the chambers at the same time. After 15–16 d, half of the plants of each plant type were moved into a similar growth chamber with similar CO_2 conditions for herbivore treatment in order to avoid any air-mediated induction of VOC synthesis in the control plants. Five individual plants per plant type and CO_2 concentration were infested with five second- to third-instar DBM larvae and additional larvae were transferred to the Bt plants after 24 h to replace any dead larvae in the first repetition. Control plants were intact plants of the same age. After 48 h, the larvae were removed from plants, and subsequently the plants were used either for collecting VOCs or for Y-tube olfactometric tests.

VOCs were collected from five individual vegetative-stage plants per treatment (two plant types; two CO_2 concentrations; herbivory/no herbivory), from a total of 40 plants per repetition. The collection system used is described elsewhere (Ibrahim *et al.*, 2005). In addition, in the VOC collections we used the same controlled CO_2 concentrations as used during growth of the plants, with the pressurized air going to the VOC collection chamber supplied from gas tanks, adjusted to 360 (control) or 720 (elevated) μl l^{-1} CO_2. The collection time was 30 min (first repetition) or 60 min (second repetition). The samples were analysed with a gas chromatograph-mass spectrometer equipped with a mass spectral detector (Hewlett-Packard GC 6890, MSD 5973; Hewlett-Packard, Palo Alto, CA, USA) as described previously (Ibrahim *et al.*, 2005) with an HP-5 column (length 50 m; internal diameter 0.2 mm; film thickness 0.5 μm) in repetition 1 and with an HP-5MS column (length 50 m; internal diameter 0.2 mm; film thickness 0.33 μm; J&W Scientific, Agilent Technologies, Santa Clara, CA, USA) in repetition 2. Identification was based on comparing mass spectra to the spectra of pure standards (Vuorinen *et al.*, 2004a; Ibrahim *et al.*, 2005) and those from the Wiley library (John Wiley & Sons, Ltd., Chichester, UK).

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Quantification was based on known concentrations of reference compounds injected into Tenax TA (Supelco, Bellefonte, PA, USA). As we had no standard for α-thujene and δ-3-carene, their responses were quantified using limonene and the responses of β-elemene and (*E*,*E*)-α-farnesene were quantified using (*E*)-β-farnesene (Vuorinen *et al.*, 2004b; Ibrahim *et al.*, 2008). Although this method has been used in numerous previous studies (e.g. Degenhardt & Lincoln, 2006; Blande *et al.*, 2007; Staudt & Lhoutellier, 2007), it does not allow 100% accuracy in quantification with mass spectrometer detection, and the quantitative amounts of these compounds are tentative. However, similar treatment effects would be seen with data based purely on relative peak areas, which is why tentative quantification was preferred to at least give an estimation of the quantitative scale. The emission was calculated as ng g^{-1} dry weight shoot biomass h^{-1} (oven-drying for 48 h at 60°C). Leaf area (cm^2 per plant) was also measured by scanning or photographing the leaves and the specific leaf area (SLA) was calculated by dividing the leaf area by the dry weight of the leaves. The number of feeding holes, grouped into larger and smaller than 2 mm diameter, on the herbivore-infested plant leaves was recorded in repetition 1. A more precise quantification of the amount of leaf area fed was achieved by photographing the herbivore-damaged leaves in repetition 2, and calculating the feeding area in mm^2 with Adobe Photoshop software (Adobe Systems Incorporated, Wilmington, DE, USA).

Female *C. vestalis* parasitoids were individually introduced into a Y-tube system described previously (Ibrahim *et al.*, 2005). Air going to the Y-tube system was adjusted to the corresponding CO_2 concentrations (supplied from gas tanks) that the plants had been grown in (i.e. control (360 μl l^{-1}) or elevated (720 μl l^{-1})). The maximum time for the parasitoid to make a choice was set to 5 min. Each parasitoid individual was tested only once. A total of 8–10 plant pairs were tested per CO_2 concentration and plant type, and 8–10 wasps were tested per plant pair over two consecutive days. Comparisons were made between transgenic (DBM infested vs noninfested) and nontransgenic (DBM infested vs noninfested) plants at control (360 μl l^{-1}) and elevated (720 μl l^{-1}) CO_2.

Expt 2: VOCs from O_3 treatments and tritrophic interactions

Plants were sown at two time-points (7-d intervals to enable testing of same-aged plants) with no O_3 (control) or chronic elevated O_3 (from the 3rd day after sowing; 100 nl l^{-1} for 8 h daily, from 08:30 to 16:30 h) in four similar growth chambers (two for control and two for elevated O_3). O_3 was generated from pure oxygen with an O_3 generator (OZ 500 Ozone Generator; Fisher, Bonn, Germany), and continuously monitored with an O_3 analyser (Model O_3 42M; Environnement S.A., Poissy, France). Two days before each VOC collection or Y-tube test day, 16–24 plants (17–26 d old) were randomly selected from the two chambers, and infested with DBM larvae for 48 h as in Expt 1. Plants with and without herbivores were placed into different chambers (having similar conditions as before, i.e. either filtered air or 100 nl l^{-1} O_3 in two chambers) to avoid air-mediated induction.

For collecting VOCs and testing parasitoid behaviour under elevated O_3, a different VOC collection and Y-tube testing system than used in Expt 1 was developed (described in detail in Pinto *et al.*, 2007b). Because of the reactive properties of O_3, a larger air space and a higher plant biomass were needed to ensure maintenance of a stable O_3 concentration inside the system. This, however, led to quantitative differences in emissions of the plants in control conditions compared with emissions from slightly younger control plants in Expt 1, and therefore the results of the two experiments are to be considered independently.

For the VOC collection, four vegetative-stage plants (19–28 d old), in pots that had the soil covered with Teflon covers, were simultaneously placed inside a 22-l glass desiccator. These four plants together served as one replicate for each treatment. In total, VOCs were collected from four replicates (16 plants per treatment; 64 in total), and 20 to 28 plants per treatment were used in the Y-tube behavioural assay. Separate plants were used in VOC sampling and the Y-tube assay. VOCs were collected under no O_3 or 100 nl l^{-1} O_3 for 90 min from each set of plants. VOCs were analysed as in Expt 1 with an HP-5MS column.

We used the same criteria in the Y-tube assay for assessing *C. vestalis* orientation as in Expt 1. In addition, as previous experience can enhance their searching efficacy (Potting *et al.*, 1999), the parasitoids were offered a learning period of 30 min by placing a DBM-infested cv. Westar oilseed rape plant inside the cage at the beginning of the day on which they were used in the Y-tube tests. Comparisons were made between transgenic (DBM infested vs noninfested) and nontransgenic (DBM infested vs noninfested) plants at control (0 nl l^{-1}; filtered air) and elevated (100 nl l^{-1}) O_3. A total of four to six sets of plants per treatment were used over 4 d.

Statistical analysis

Before statistical analysis, we tested the data set for normality and equality of error variances of variable residuals. As a result, some variables were log (x + 1) or square-root transformed for normality. Biomass and specific leaf area results were analysed with a linear mixed model (plant type and CO_2 concentration as fixed effects and the repetition of the experiment as a random effect). Herbivore feeding damage results were analysed with analysis of variance with plant type and CO_2 concentration as fixed factors.

A linear mixed model with repetition of the experiment as a random factor (CO_2 experiment) or analysis of variance (O_3 experiment) was used in further analysis. VOC results from the CO_2 experiments were analysed first using a preplanned comparison between intact non-Bt and Bt plants in the control treatment. Further models included: plant type and

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Table 1 Shoot biomass ± 1 SEM and specific leaf area (SLA) ± 1 SEM in nontransgenic (Non-Bt) cv. Westar and Bt-transgenic (Bt) oilseed rape (*Brassica napus* ssp. *oleifera*) plants grown under control or elevated CO_2 and under no ozone (control, filtered air) or elevated ozone and the results of the statistical analysis[a]

	Shoot biomass (mg DW)		SLA (cm^2 g^{-1} DW)	
	Non-Bt	Bt	Non-Bt	Bt
Control CO_2	200.5 ± 27.1	161.3 ± 14.6	398.2 ± 15.2	394.5 ± 20.6
Elevated CO_2	255.4 ± 23.9	201.8 ± 23.4	356.6 ± 14.5	351.4 ± 15.4
	$F_{1,36}$	P	$F_{1,36}$	P
Plant type	4.169	**0.049**	0.123	0.728
CO_2 concentration	4.406	**0.043**	10.950	**0.002**
Plant type × CO_2 concentration	0.100	0.754	0.004	0.953
Control, no O_3	282.0 ± 32.3	171.3 ± 14.2		
100 nl l^{-1} O_3	201.6 ± 23.4	112.3 ± 15.2		
	$F_{1,8}$	P		
Plant type	19.788	**0.002**		
O_3	9.627	**0.015**		
Plant type × O_3	0.225	0.648		

[a]Plants were grown under the following conditions. Expt 1: 360 µl l^{-1} (control) or 720 µl l^{-1} (elevated) CO_2, with plants harvested at age 17–18 d (two repetitions, $n = 10$, individual plants used as replicates). Expt 2: no O_3 (control) or 100 nl l^{-1} O_3 for 8 h daily, with plants harvested at age 19–21 d ($n = 3$, four plants per replicate). ANOVA or mixed model results for main effects of plant type, CO_2 concentration (CO_2) and elevated O_3 (O_3) and their interactions are shown (statistical significance ($P < 0.05$) in bold). DW, dry weight.

herbivory among CO_2 or O_3 treatments (to assay herbivore induction); plant type and CO_2 or O_3 treatment excluding herbivory (to analyse CO_2 and O_3 effects on constitutive VOCs); and finally plant type, CO_2 or O_3 treatment and herbivory to compare all treatment means within experiments. The Student–Newman–Keuls test was used for post hoc comparisons of VOC results. Emissions of α-thujene, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (*E*,*E*)-α-farnesene in the O_3 experiment were analysed with the nonparametric Kruskal–Wallis test followed by Mann–Whitney *U* post hoc tests with Bonferroni correction, because of failure to meet the predictions of parametric tests. Behavioural tests for *C. vestalis* were analysed with the nonparametric binomial test. All statistical analyses were performed using the SPSS for Windows 14.0 statistical software package (SPSS Inc., Chicago, IL, USA).

Results

For plants of both types (i.e. nontransgenic and Bt-transgenic), shoot biomass was higher in plants grown under elevated CO_2 than in those grown under control CO_2 (Table 1). The SLA was reduced by elevated CO_2. Chronic 100 nl l^{-1} O_3 treatment reduced shoot biomass. Bt plants had a lower total vegetative biomass than non-Bt plants in both of the experiments, but the response to CO_2 or O_3 elevations was similar in the two plant types. Chronic O_3 exposure caused slight visible damage on the leaves of both plant types, which appeared as interveinal chlorosis (data not shown).

DBM larvae fed successfully, in a continuous manner, on the non-Bt plants, whereas the larvae were able only to initiate feeding on Bt plants. The number of feeding holes > 2 mm diameter was 9.2 ± 1.0 (mean ± 1 SEM) in nontransgenic plants and zero in Bt plants (ANOVA, $F_{1,16} = 240.3$, $P < 0.001$ for main effect of plant type). There were 1.8 ± 1.1 and 17.2 ± 3.5 feeding holes < 2 mm diameter in the nontransgenic and Bt-transgenic plants under control CO_2 (ANOVA, $F_{1,16} = 45.08$, $P < 0.001$ for main effect of plant type). The leaf area eaten was 27.6 ± 5.7 and 3.6 ± 0.5 mm^2 for the nontransgenic and Bt-transgenic plants (ANOVA, $F_{1,16} = 55.58$, $P < 0.001$, main effect of plant type), respectively. Elevated CO_2 did not statistically significantly affect the number of feeding holes or the amount of leaf area fed ($P > 0.05$ for main effect of CO_2 concentration). There were 9.8 ± 0.6 and 0.4 ± 0.4 feeding holes > 2 mm diameter and 2.8 ± 1.2 and 13.8 ± 0.9 feeding holes < 2 mm diameter (representing total feeding areas of 33.4 ± 4.7 and 2.2 ± 0.4 mm^2) in the non-Bt and Bt plants under elevated CO_2, respectively.

We quantified terpenoid emissions in the VOC profiles of nontransgenic and Bt-transgenic plant types grown with CO_2 or O_3 enhancement and in the corresponding control conditions in both experiments (Table 2). In addition to terpenoids, one green leaf volatile (GLV), (*Z*)-3-hexenyl acetate, was detected from the emission profile, but its emission was not significantly affected by plant type, elevated CO_2 or O_3, or herbivory (results not shown). We also detected small quantities of glucosinolate breakdown products, such as isothiocyanates,

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Table 2 Terpenoid emissions from intact and *Plutella xylostella* (diamondback moth (DBM))-damaged nontransgenic (Non-Bt) and Bt-transgenic (Bt) oilseed rape (*Brassica napus* ssp. *oleifera*) plants grown in (a) control CO_2 or elevated CO_2 and in (b) no ozone (control, filtered air) or elevated ozone

(a) Terpenoid (ng g^{-1} h^{-1} shoot DW)	Control CO_2 (360 µl l^{-1})				Elevated CO_2 (720 µl l^{-1})			
	Non-Bt	Non-Bt + DBM	Bt	Bt + DBM	Non-Bt	Non-Bt + DBM	Bt	Bt + DBM
α-Thujene[t]	2.82 ± 0.85[a]	6.52 ± 0.93[b]	2.60 ± 0.90[a]	4.98 ± 1.24[ab]	5.83 ± 2.05[a]	14.96 ± 2.82[b]	6.61 ± 2.22[a]	10.49 ± 2.13[ab]
α-Pinene	4.84 ± 1.24	4.70 ± 0.58	1.88 ± 0.68	4.26 ± 0.82	3.49 ± 0.95[a]	9.48 ± 1.00[b]	4.62 ± 1.67[a]	7.52 ± 1.58[ab]
Sabinene	15.65 ± 4.62[a]	32.21 ± 4.54[b]	25.44 ± 1.43[ab]	34.49 ± 4.81[b]	40.01 ± 3.66[a]	72.74 ± 7.86[b]	51.15 ± 7.43[ab]	68.55 ± 7.53[b]
β-Myrcene	8.97 ± 2.14[ab]	10.36 ± 1.39[b]	3.79 ± 1.31[a]	9.40 ± 1.60[ab]	8.72 ± 2.20[a]	21.81 ± 2.80[b]	8.08 ± 2.78[a]	15.70 ± 2.31[ab]
δ-3-Carene[t]	0.74 ± 0.74	2.41 ± 2.41	7.55 ± 4.62	8.20 ± 4.39	10.56 ± 4.31	11.98 ± 4.89	nd	16.11 ± 5.02
Limonene	17.96 ± 2.28[a]	29.78 ± 4.53[b]	16.12 ± 2.86[a]	29.04 ± 3.48[b]	27.20 ± 6.71[a]	66.50 ± 10.72[b]	31.14 ± 7.65[a]	50.55 ± 8.87[ab]
1,8-Cineole	38.54 ±11.58	64.33 ± 19.67	34.20 ± 14.40	48.78 ± 18.66	81.09 ± 27.71	138.41 ± 45.71	98.87 ± 33.00	126.67 ± 38.02
γ-Terpinene	0.62 ± 0.40	1.51 ± 0.56	0.38 ± 0.38	0.46 ± 0.46	3.05 ± 1.52	3.79 ± 1.58	2.42 ± 0.44	3.30 ± 0.68
(*E*)-DMNT	1.66 ± 1.50[a]	18.65 ± 5.87[b]	0.35 ± 0.24[a]	0.92 ± 0.43[a]	1.16 ± 0.69[a]	25.76 ± 5.17[b]	1.71 ± 1.12[a]	2.65 ± 1.38[a]
β-Elemene[t]	2.76 ± 2.76	11.86 ± 5.79	nd	9.18 ± 3.76	1.19 ± 1.19[a]	26.93 ± 4.58[c]	nd[a]	18.34 ± 0.97[b]
(*E*,*E*)-α-Farnesene[t]	4.75 ± 1.42	10.39 ± 2.16	5.75 ± 1.73	5.55 ± 1.88	7.04 ± 0.99[a]	13.85 ± 0.88[b]	4.63 ± 1.36[a]	4.41 ± 1.12[a]
Total	87.06 ± 17.82[a]	163.54 ± 36.71[b]	78.50 ± 24.58[a]	126.33 ± 33.52[ab]	158.40 ± 49.95[a]	341.57 ± 82.33[c]	180.13 ± 57.77[ab]	268.93 ± 71.54[bc]
(b) Terpenoid (ng g^{-1} h^{-1} shoot DW)	Control (filtered air, no ozone)				Elevated ozone (100 nl l^{-1})			
	Non-Bt	Non-Bt + DBM	Bt	Bt + DBM	Non-Bt	Non-Bt + DBM	Bt	Bt + DBM
α-Thujene[t]	0.14 ± 0.14	0.49 ± 0.29	1.11 ± 0.98	1.11 ± 0.91	nd	nd	nd	nd
α-Pinene	3.65 ± 0.55	5.19 ± 1.28	6.27 ± 1.09	8.79 ± 2.31	3.92 ± 1.25	3.20 ± 1.16	5.37 ± 3.00	5.90 ± 2.71
Sabinene	1.16 ± 0.15[a]	3.40 ± 0.65[b]	1.67 ± 0.28[a]	3.12 ± 0.29[b]	0.78 ± 0.13	2.25 ± 0.23	1.67 ± 0.74	1.95 ± 0.62
β-Myrcene	2.21 ± 0.43	3.35 ± 0.68	2.53 ± 0.53	4.05 ± 0.89	0.78 ± 0.58	2.46 ± 1.11	2.84 ± 1.91	0.59 ± 0.59
δ-3-Carene[t]	1.72 ± 0.17[a]	2.02 ± 0.20[a]	3.39 ± 0.43[b]	3.75 ± 0.52[b]	0.74 ± 0.39	1.13 ± 0.56	1.91 ± 0.20	0.28 ± 0.28
Limonene	5.16 ± 1.97	7.64 ± 3.37	8.60 ± 3.04	9.56 ± 3.12	2.76 ± 1.25	3.30 ± 0.95	6.42 ± 2.03	5.39 ± 1.83
1,8-Cineole	1.43 ± 0.27	2.27 ± 0.24	2.54 ± 0.72	3.14 ± 0.93	2.72 ± 1.78	3.63 ± 1.43	2.40 ± 1.00	2.61 ± 1.17
γ-Terpinene	nd	nd	nd	nd	nd	nd	nd	nd
(*E*)-DMNT	nd	1.10 ± 0.20	nd	nd	nd	0.08 ± 0.08	nd	nd
β-Elemene[t]	nd	nd	nd	nd	nd	nd	nd	nd
(*E*,*E*)-α-Farnesene[t]	nd	1.24 ± 0.53	0.15 ± 0.15	0.17 ± 0.17	nd	nd	nd	nd
Total	15.46 ± 2.34	26.69 ± 4.83	26.26 ± 4.61	33.69 ± 6.34	11.70 ± 3.44	16.05 ± 3.90	20.62 ± 7.81	16.71 ± 5.41

DBM-damaged plants were infested for 48 h before volatile organic compound (VOC) collection. VOCs were collected under the same CO_2 and O_3 concentrations under which the plants were growing. Values correspond to the average of four (O_3 experiment) and five to ten replicates (CO_2 experiment) ± 1 SEM. Different superscript letters in the results indicate statistically significant (Student–Newman–Keuls test, $P < 0.05$) differences in treatment means (non-Bt and Bt plants with or without herbivory) analysed within each CO_2 or O_3 treatment. [t]Tentative quantification; no authentic standard compound available; quantification based on response of structurally most related terpenoid. nd, not detected (below detection limit); DMNT, (*E*)-4,8-dimethyl-1,3,7-nonatriene; DW, dry weight.

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Table 3 *P*-values for main effects of CO_2 concentration, elevated O_3 and herbivory (*Plutella xylostella* feeding) and their interactions on emissions of individual terpenoids[a]

(a) Source	α-Thu	α-Pin	Sab	β-Myr	δ-Car	Lim	Cin	γ-Ter	DMNT	β-Ele	α-Far	Total
CO_2	< **0.001**	**0.002**	< **0.001**	< **0.001**	0.075	< **0.001**	< **0.001**	**0.001**	0.242	**0.016**	0.418	< **0.001**
Her	< **0.001**	< **0.001**	< **0.001**	< **0.001**	0.073	< **0.001**	**0.006**	0.312	< **0.001**	< **0.001**	**0.008**	< **0.001**
Bt × CO_2	0.530	0.377	0.751	0.904	0.085	0.494	0.561	0.945	0.669	0.628	0.069	0.950
Bt × Her	**0.035**	0.842	0.162	0.805	0.211	0.176	0.362	0.790	< **0.001**	0.415	**0.005**	0.136
CO_2 × Her	**0.027**	**0.025**	0.134	**0.008**	0.165	**0.016**	0.317	0.798	0.334	**0.006**	0.786	0.075
Bt × CO_2 × Her	0.203	0.057	0.627	0.056	0.152	0.131	0.681	0.703	0.381	0.404	0.779	0.424

(b) Source	α-Thu	α-Pin	Sab	β-Myr	δ-Car	Lim	Cin	γ-Ter	DMNT	β-Ele	α-Far	Total
O_3	na	0.168	**0.033**	0.385	< **0.001**	0.072	0.156	nd	na	nd	na	**0.014**
Her	na	0.397	< **0.001**	0.255	0.376	0.675	0.976	nd	na	nd	na	0.182
Bt × O_3	na	0.520	0.584	0.572	**0.015**	0.955	0.624	nd	na	nd	na	0.559
Bt × Her	na	0.625	0.090	0.551	**0.039**	0.658	0.506	nd	na	nd	na	0.393
O_3 × Her	na	0.350	0.097	0.619	**0.043**	0.575	0.580	nd	na	nd	na	0.202
Bt × O_3 × Her	na	0.955	0.744	0.405	**0.031**	0.994	0.563	nd	na	nd	na	0.750

[a]CO_2 control (360 μl l^{-1}) vs elevated (720 μl l^{-1}) CO_2 concentration; Herbivory (Her), intact vs DBM damaged; Bt, non-Bt vs Bt; O_3, control (filtered air) vs elevated (100 nl l^{-1}) O_3. α-Thu, α-thujene; α-Pin, α-pinene; Sab, sabinene; β-Myr, β-myrcene; δ-Car, δ-3-carene; Lim, limonene; Cin, 1,8-cineole; γ-Ter, γ-terpinene; DMNT, (*E*)-4,8-dimethyl-1,3,7-nonatriene; β-Ele, β-elemene; α-Far, (*E*,*E*)-α-farnesene; nd, not detected; na, not applicable (variable does not allow ANOVA).
Statistical testing is based on (a) $F_{1,71}$, linear mixed model and (b) $F_{1,24}$, ANOVA; significant effects ($P < 0.05$) in bold.

thiocyanates and nitriles, from the VOC profile (results not shown). However, because of their very low concentrations, we were unable to effectively quantify their emissions.

In the CO_2 experiment, the first preplanned comparison between intact non-Bt and Bt plants of the control treatment revealed no significant differences ($P > 0.05$) in constitutive emissions between the plant types (Table 2a). Total terpenoid emission was increased by herbivory in the control CO_2 treatment (mixed model including plant type and herbivory under control CO_2, $F_{1,35} = 12.63$, $P = 0.001$), and under elevated CO_2 (mixed model with plant type and herbivory under elevated CO_2, $F_{1,35} = 29.36$, $P < 0.001$). Elevated CO_2 increased total emission from intact plants (mixed model including intact plants of both plant types, $F_{1,35} = 4.43$, $P = 0.042$). For individual terpenoids, elevated CO_2 increased emissions of α-thujene, sabinene, limonene, 1,8-cineole and γ-terpinene from intact non-Bt and Bt plants (mixed model including intact plants under both CO_2 concentrations, $P < 0.05$, results not shown). Under control CO_2, DBM herbivory led to increased emissions of α-thujene, sabinene, β-myrcene, limonene and β-elemene in both plant types, and also of DMNT in non-Bt plants (mixed model including plants grown under control CO_2, $P < 0.05$, results not shown). In the elevated CO_2 treatment, emissions of all the aforementioned compounds were increased by DBM herbivory. In addition, α-pinene and δ-3-carene emissions were higher after herbivory in both plant types and also DMNT and (*E*,*E*)-α-farnesene emissions in the non-Bt plants (mixed model including plants grown under elevated CO_2, $P < 0.05$, results not shown). The statistical model including both CO_2 and herbivory treatments showed significant interactions between elevated CO_2 and herbivory as an indication of significantly higher increases in α-thujene, α-pinene, β-myrcene, limonene and β-elemene emissions under elevated CO_2 in both plant types (Table 3a). The statistically significant interactions between plant type and herbivory indicated higher induction of DMNT in particular, and also of (*E*,*E*)-α-farnesene and α-thujene, from the non-Bt plants than from the resistant Bt-transgenic plants (Table 3a). No plant type × CO_2 interactions were observed (Table 3a), which indicates that the response to elevated CO_2 was similar in the two plant types.

In the O_3 experiment, the preplanned comparison between intact non-Bt and Bt plants revealed a higher emission of δ-3-carene from the Bt plants ($F_{1,12} = 2.75$, $P = 0.011$). In a comparison of plant type and O_3 in intact plants, δ-3-carene emission was found to be reduced by O_3 (ANOVA, main effect of O_3, $F_{1,12} = 12.98$, $P = 0.004$), and was higher in intact elevated O_3-grown Bt than non-Bt plants (ANOVA, main effect of plant type, $F_{1,12} = 17.47$, $P = 0.002$). When all the studied factors were included in the statistical model, there was a significant interaction of plant type × O_3 × DBM feeding (ANOVA, $F_{1,24} = 5.27$, $P = 0.031$) for δ-3-carene emissions (Table 3b). When individual treatments were compared, this seemed to correspond to the decrease in δ-3-carene emission

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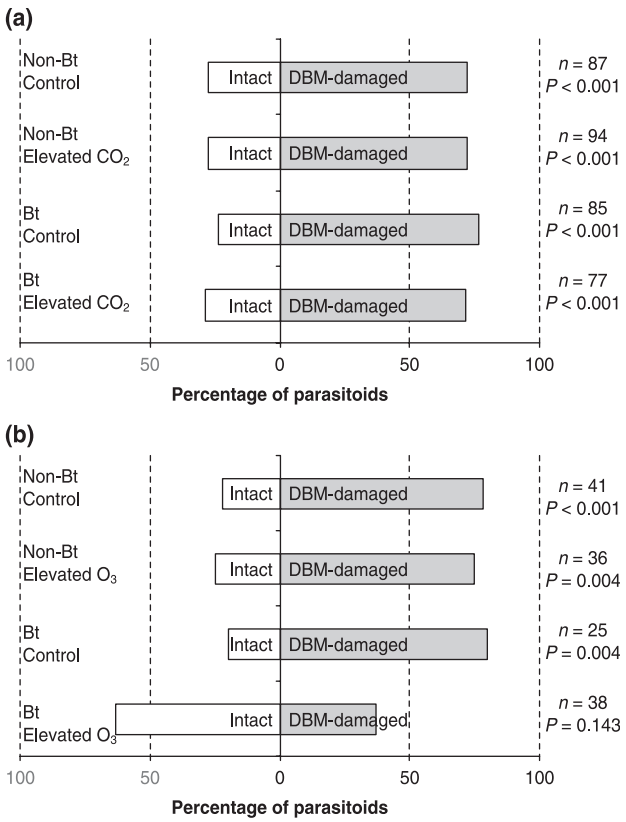

Fig. 1 Orientation of *Cotesia vestalis* parasitoids in the Y-tube olfactometer to intact versus *Plutella xylostella* (diamondback moth (DBM))-damaged nontransgenic (Non-Bt) and Bt-transgenic (Bt) oilseed rape (*Brassica napus* ssp. *oleifera*) plants. Plants were grown and the behavioural tests conducted under the following CO_2 and O_3 concentrations: (a) CO_2 experiment: control CO_2, 360 μl l^{-1} CO_2; elevated CO_2, 720 μl l^{-1} CO_2; (b) O_3 experiment: control, filtered air, 0 nl l^{-1}; elevated O_3, 100 nl l^{-1}. *n* represents the total number of parasitoids making a choice, excluding no choices. *P*-values < 0.05 indicate significant difference from the 0.5 distribution (nonparametric binomial test, exact).

observed in Bt plants after DBM feeding compared with intact Bt plants in elevated O_3 (Table 2b). The emission of the terpenoid sabinene increased after DBM feeding (ANOVA including all factors, main effect of DBM feeding, $F_{1,24} = 21.74$, $P < 0.001$), and was reduced in elevated O_3 (main effect of O_3, $F_{1,24} = 5.39$, $P = 0.030$) (Tables 2b, 3b). Total terpenoid emissions were also reduced by elevated O_3 (main effect of O_3, $F_{1,24} = 7.14$, $P = 0.014$). Post hoc comparisons including both plant types under control conditions revealed a significant increase of sabinene emitted after herbivory in both plant types. Under elevated O_3, the intact and DBM-damage treatment groups did not differ statistically significantly (Table 2). DBM damage induced DMNT emission from individual nontransgenic plants, whereas this compound was not emitted in a quantifiable amount from Bt plants. Also, overall the emissions were low compared with DMNT emissions in the CO_2 experiment and lacked statistical significance (nonparametric Mann–Whitney *U* test) in this experiment. This can be partly attributed to the larger air space used in the collection system with O_3, which also led to emissions of β-elemene and γ-terpinene falling below the quantification limit. In other respects, non-Bt and Bt plants or plants grown under no O_3 and under elevated O_3 did not exhibit any significant differences in terpenoid emissions.

Under both control CO_2 and elevated CO_2 conditions (Expt 1), host-damaged plants attracted naïve *C. vestalis* in both plant types (Fig. 1a). The percentage of parasitoids orientating to DBM-damaged plants was 72.4 and 72.3% for non-Bt plants, and 76.5 and 71.4% for Bt-transgenic plants under control and elevated CO_2, respectively. Similarly, in the O_3 experiment, experienced *C. vestalis* preferred the odour of host-damaged Bt plants ($P = 0.004$) and non-Bt plants ($P < 0.001$) over that of the corresponding intact plants in the absence of O_3 (Fig. 1b) (78.0% of parasitoids orientated to non-Bt and 80.0% to Bt-transgenic damaged plants). *Cotesia vestalis* preferred host-damaged non-Bt plants to intact non-Bt plants under elevated O_3 (75.0% orientated to damaged plants), whereas there was no difference in the choices of the parasitoids between intact and host-damaged Bt plants in elevated O_3 ($P = 0.143$; 36.8% of parasitoids orientated to damaged plants versus 63.2% to undamaged plants) (Fig. 1b).

Discussion

The qualitative VOC profiles of intact Bt and non-Bt plants, and the physiological responses and individual terpenoid responses of the plant types to elevated CO_2 or elevated O_3 were similar, which indicated that Bt production does not interfere with allocation of resources to the major pathways leading to VOC emissions in control conditions, or under these abiotic changes, in the vegetative stage in oilseed rape. Here, the Bt-transgenic plants showed slight developmental delays in which phenology and vegetative biomass lagged behind those of the nontransgenic plants, a finding that was similar to the results of our earlier studies on these lines (Himanen *et al.*, 2008a,b). Higher δ-3-carene emissions in the O_3 experiment in Bt versus non-Bt plants might be explained by the earlier developmental stage of the Bt plants, which could lead to higher emission capacity. This observation reveals the need for a further long-term, preferably seasonal comparison of the VOC profiles of these plant types. Yan *et al.* (2004) found quantitative differences between Bt and non-Bt cotton plants, but they also did not perform a seasonal assessment. Previously, oviposition by DBM females was found to be similar on nontransgenic versus Bt-transgenic canola (*B. napus*; Ramachandran *et al.*, 1998), cabbage (*B. oleracea* ssp. *capitata*; Kumar, 2004), and broccoli (Tang *et al.*, 1999), which suggested that intact non-Bt and Bt plants yield equivalent olfactory stimuli to DBM. In these studies there were, unfortunately, no analyses of VOCs. Other factors, such

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as adequate contact stimuli, can also have major effects on DBM oviposition (Talekar & Shelton, 1993; Spencer *et al.*, 1999), and therefore equality of oviposition does not directly indicate similarity in VOC emissions. Recently, Bt-transgenic varieties have been tested as potential trap crops for pests such as DBM (Shelton *et al.*, 2008), and in this respect equal volatile emissions from intact plants are also important to ensure efficient attraction of pest females.

Significant quantitative differences in herbivore-inducible emissions have been found in comparisons between Bt-transgenic plants and their parent lines in maize (Turlings *et al.*, 2005; Dean & De Moraes, 2006) and oilseed rape (Ibrahim *et al.*, 2008), in which there were differences in herbivore feeding type or quantity. When Bt and non-Bt maize plants suffered identical amounts of herbivore damage, or mechanical damage and applied larval regurgitant, there were similar VOC emissions between the plant types (Dean & De Moraes, 2006), which indicated a significant role of feeding damage in induction rather than proposed allocation differences. Turlings *et al.* (2005) reported that seven compounds, including the two homoterpenes DMNT and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT), were emitted in lower amounts from damaged Bt maize plants compared with identically treated non-Bt maize plants. Comparing herbivore-induced emissions on non-Bt and Bt oilseed rape, Ibrahim *et al.* (2008) found that DMNT, a compound whose emission is expected to be strongly induced by herbivory in *Brassica* (Vuorinen *et al.*, 2004a,b), and (*E*,*E*)-α-farnesene were released in smaller amounts from Bt oilseed rape. Emissions of the same two compounds were also induced less strongly in Bt than non-Bt plants in the current study under elevated CO_2, and in the case of DMNT also under control CO_2. The system used for O_3 exposure clearly led to lower emissions available for detection, and presumably therefore this effect was not significant in that experiment. Bt toxin produced a large reduction in herbivory in Bt oilseed rape plants, but some damage did occur, resulting in numerous small holes in Bt plants, which led to induced emissions of most of the terpenoids. Based on the current and previous (Turlings *et al.*, 2005; Ibrahim *et al.*, 2008) results, DMNT emission in particular requires continuous successful feeding. Considering the capabilities of Bt plants to maintain efficient indirect defences, it is highly desirable that the transgenic trait allows plants to induce terpenoid emissions, that is, minor plant damage is advantageous. The ability of Bt to protect plants directly and also allow indirect defence seems to represent the best of both worlds.

Doubled atmospheric CO_2 significantly increased terpenoid emissions from intact oilseed rape plants compared with those released from the corresponding plants grown in control CO_2, when measured per dry weight or on the basis of leaf area (data not shown). As elevated CO_2 typically has both physiological effects (i.e. it increases the thickness of leaves (also shown as reduced SLA in this study), decreases stomatal density and alters stomatal function (Niinemets *et al.*, 2004; Vuorinen *et al.*, 2004a)) and biochemical effects (such as competition for pyruvate which is needed for higher rates of photosynthesis (Rosenstiel *et al.*, 2003)) on VOC emissions, based on previous knowledge the emission of VOCs could have been expected to decline with CO_2 elevation (Loreto *et al.*, 2001; Rosenstiel *et al.*, 2003). However, it is important to note that here we used only early vegetative-stage plants to detect compounds meaningful for tritrophy. The plants grown under elevated CO_2 had higher total biomass and leaf numbers (results not shown) compared with control CO_2-grown plants, which could lead to differences in emissions based on plant and leaf developmental stage (i.e. phenological differences). Although the intrinsic release of VOCs through increased leaf thickness and stomatal function would have actually been decreased by CO_2 elevation (Niinemets *et al.*, 2004), the higher number of photosynthetically active leaves could result in higher emissions, as we detected under elevated CO_2. It is known that leaf age influences VOC emissions, and younger, actively photosynthesizing leaves typically have the highest VOC synthesis levels and induction capabilities (Takabayashi *et al.*, 1994; Gouinguené & Turlings, 2002). Therefore, assessing the effects of phenological changes over a longer timescale seems reasonable in order to reveal the seasonality in VOC emission responses, as these were clearly influenced by elevated CO_2 in young oilseed rape plants.

Herbivore-inducible VOC emissions were even higher from plants grown under elevated CO_2 than from those grown under control CO_2, indicating that elevated CO_2 conditions could modify herbivore-induced defences at the vegetative stage, and enhance indirect defence in the future. The degree of feeding damage by DBM larvae did not differ statistically significantly between the CO_2 treatments, so the higher induction of VOCs by herbivory in elevated CO_2 is not attributable to increased (i.e. compensatory) feeding (Docherty *et al.*, 1996), but is a plant-mediated effect.

Allocation of resources for defence under O_3 exposure is less clear in theory compared with the effects induced by elevated CO_2, as commonly explained through changes in carbon and nitrogen dynamics (Herms & Mattson, 1992). O_3 activates defence signalling and the synthesis of defence-related proteins, and at the same time it damages existing tissues and O_3-sensitive proteins (Fiscus *et al.*, 2005). Acute O_3 exposure has previously been found to increase emissions of methyl salicylate, sesquiterpenes and GLVs in a number of plant species (Heiden *et al.*, 1999; Wildt *et al.*, 2003; Beauchamp *et al.*, 2005), as well as the emission of homoterpenes from lima bean (*Phaseolus lunatus*; Vuorinen *et al.*, 2004c). In the current study, no O_3 damage-induced increases in VOC emissions were observed under chronic 100 ppb O_3, corresponding to concentrations found typically in only severely polluted areas (IPCC, 2007). However, as important for ecological interactions, most former studies have not taken into account the fact that O_3 and VOCs can react in the

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atmosphere to form secondary aerosols, which leads to a significant loss of biogenic VOCs (McFrederick *et al.*, 2008), and decreases cues for multitrophic signalling in elevated O_3 environments (Pinto *et al.*, 2007a,b,c). Our set-up does not distinguish between plant-mediated effects of O_3 on VOC emission profile and gas phase reactions of VOCs with O_3. Some VOC emissions can already be induced or repressed by chronic O_3 during the growth of plants, whereas their reactivity in the atmosphere is compound-specific, which complicates summarizing the overall change in VOC emissions caused by O_3. If plant-mediated changes predominate, more dramatic and plant species-variable changes in VOC signalling could be predicted, which would be more unpredictable in natural conditions. However, if gas-phase reactions with O_3 lead to a lower VOC abundance, these changes could be more easily predicted, as they should mainly follow chemical reaction pathways (Atkinson & Arey, 2003). Parasitoids could more readily adapt to plant-mediated changes, as these would not be rapid, in contrast to O_3 reactivity, which would lead to sudden changes and might therefore severely confuse orientation of some species.

There is limited knowledge of VOC responses of crop species to O_3 (Pinto *et al.*, 2007b), but our results are in accordance with those of recent field studies on birch (*Betula pendula*) and aspen (*Populus tremula* × *tremuloides*) trees (Vuorinen *et al.*, 2005; Blande *et al.*, 2007) in which it was reported that 1.4–2 times ambient chronic O_3 elevation leads to only very small changes in terpenoid emissions. Pinto *et al.* (2007a,b,c) have demonstrated in laboratory conditions that most, but not all, plant-emitted terpenoids are degraded by acute O_3 targeted to ambient ozone-grown plants. The increase of 1,8-cineole emission as a percentage of total emission with O_3 elevation in our current study confirmed the previously reported tolerance of this compound to O_3, as a result of its chemical structure, in comparison to most other terpenoids vulnerable to O_3 degradation (Pinto *et al.*, 2007a). Such O_3-tolerant compounds might play a major role in the third trophic level, by allowing some cues to remain unaffected.

Bt toxin production did not confound the tritrophic signalling system in oilseed rape, *P. xylostella* and *C. vestalis* under control or elevated CO_2. In spite of the reduced DMNT and (*E*,*E*)-α-farnesene emissions, the induction of other terpenoids seemed to be sufficient as an olfactory cue for *C. vestalis* compared with intact plants. Previously, parasitoid attraction by plant-emitted VOCs has been shown to be unaffected by Bt production in maize; that is, damaged nontransgenic and transgenic plants were comparably attractive to *C. marginiventris* and *M. rufiventris* parasitoids (Turlings *et al.*, 2005). However, Schuler *et al.* (1999a) reported greater attraction of *C. plutellae* to non-Bt oilseed rape compared with Bt oilseed rape with host feeding. Again, this was related to feeding damage, as feeding of Bt-resistant larvae returned the attractiveness of Bt oilseed rape to the level of nontransgenic plants (Schuler *et al.*, 1999a).

Electrophysiological studies have shown that *Cotesia* species respond to over 20 different VOCs (Smid *et al.*, 2002; Gouinguené *et al.*, 2005). The emissions measured in the two separate experiments and in the CO_2 and O_3 treatments also showed quantitative variation in the VOC emissions with changes in the ratios of the compounds (influenced by, for example, phenological effects), yet this specialist parasitoid was able to recognize host-damaged non-Bt plants in all tested combinations, revealing a high olfactory capacity. Interestingly, in all tested comparisons, *C. vestalis* was more attracted to the treatment releasing higher total emissions, which could also indicate quantity-based preference for plant-based VOCs. Previous experience with host-damaged plants does not seem compulsory for *C. vestalis*, as their orientation in control conditions was equally successful in the CO_2 experiment (unexperienced wasps) and in the O_3 experiment (experienced wasps). Among individual terpenoids, the monoterpene sabinene may be the most important volatile cue for *C. vestalis* in the current context, as the compound clearly responded to herbivore feeding in both experiments and appeared in lower concentration under elevated O_3. The tritrophic interaction system studied here in oilseed rape, similarly to that in *B. oleracea* (Pinto *et al.*, 2007b,c, 2008), did not seem to be in jeopardy even in elevated tropospheric O_3 episodes. Therefore, even if emissions of major terpenoids are reduced by O_3, *C. vestalis* can rely on the unaffected compounds such as 1,8-cineole or other compounds that we were not able to detect here, such as glucosinolate breakdown products (Pinto *et al.*, 2007c).

However, O_3 has the potential to affect tritrophic signalling, especially if feeding damage is low, which obviously decreases inducible VOC emissions. Bt plants damaged by Bt-sensitive insect hosts did not attract *C. vestalis* under elevated O_3 here. It could be speculated that this could perhaps result in decreased parasitoid abundance around Bt plant fields (Schuler *et al.*, 2003), especially in O_3-polluted areas, which might have agro-ecological consequences. This emphasizes the need for non-Bt refugee areas not only to limit the evolution of resistance (Bates *et al.*, 2005; Tabashnik *et al.*, 2008) but also to maintain natural enemy populations. In addition, *C. vestalis* can parasitize, but not develop inside, Bt-susceptible hosts on Bt-transgenic plants, because of the detrimental effects of Bt toxin on the host larva (Schuler *et al.*, 2003, 2004). Therefore, sustainable parasitoid populations in the long term might be negatively affected if they are attracted to Bt plants, but have no suitable host for reproduction. In addition, parasitoids might learn to connect VOC cues from Bt plants to a negative response (if the majority of host larvae present are already dead as a result of Bt toxin), and lead to reduced attractiveness of Bt plants in field conditions. However, these negative effects of reduced host abundance will be equal for chemical control and use of transgenic Bt plants. Importantly, the real ecological effects of disturbance of this individual tritrophic interaction in agroecosystems containing Bt crops, compared with chemical insecticides or Bt biopesticide

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applications, might be minimal. Chemical pest control methods are not 100% effective and allow VOC induction, but they do often harm nontarget insects, including parasitoids, directly (Romeis *et al*., 2006).

Finally, even if the orientation of *C. vestalis* to its host using plant-emitted VOCs is not directly altered by elevated CO_2 or O_3, host-mediated effects through altered development times and biomass on parasitoid populations can also be significant (Percy *et al*., 2002; Holton *et al*., 2003). The complexity of the tritrophic systems present in natural ecosystems calls for studies to examine parasitism rate and abundance together with plant phenology and herbivore dynamics under elevated CO_2 and O_3, singly and in combination. This could help to determine the possible connections of parasitoid abundance with concurrent changes in VOC emissions in agroecosystems with traditional or transgenic plants: a challenging but important task for future research.

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