

Minor effects of two elicitors of insect and pathogen resistance on volatile emissions and parasitism of *Spodoptera frugiperda* in Mexican maize fields

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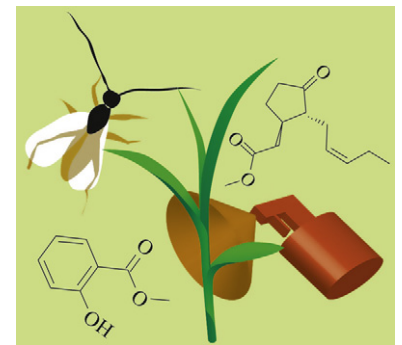
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ABSTRACT

Synthetic elicitors can be used to induce resistance in plants against pathogens and arthropod herbivores. Such compounds may also change the emission of herbivore-induced plant volatiles, which serve as important cues for parasitic wasps to locate their hosts. Therefore, the use of elicitors in the field may affect biological control of insect pests. To test this, we treated maize seedlings growing in a subtropical field in Mexico with methyl jasmonate (MeJA), an elicitor of defense responses against many insects, and benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH), an elicitor of resistance against certain pathogens. Volatile emission, herbivore infestation, pathogen infection, and plant performance (growth and grain yield) of treated and untreated maize plants were measured. Application of BTH slightly reduced volatile emission in maize, while MeJA increased the emission compared to control treatments. Despite the apparent changes in volatile emissions, the elicitor application did not consistently affect infestation by *Spodoptera frugiperda* larvae, the main insect pest found on the maize seedlings, and had only marginal effects on parasitism rates. Similarly, there were no treatment effects on infestation by other herbivores and pathogens. Results for the six replications that stretched over one summer and one winter season were highly variable, with parasitism rates and the species composition of the parasitoids differing significantly between seasons. This variability, as well as the severe biotic and abiotic stresses on young seedlings might explain why we measured only slight effects of elicitor application on pest incidence and biological control in this specific field study. Indeed, an additional field experiment under milder and more standardized conditions revealed that BTH induced significant resistance against *Bipolaris maydis*, a major pathogen in the experimental maize fields. Similar effects can be expected for herbivory and parasitism rates.

GRAPHICAL ABSTRACT



Keywords:

BTH, Methyl jasmonate, *Zea mays*, *Spodoptera frugiperda*, *Bipolaris maydis*, *Chelonus insularis*, *Campoletis sonorensis*, *Cotesia marginiventris*, *Eiphosoma vitticolle*, *Pristomerus spinator*, *Chelonus cautus*, Tachinidae, Parasitoids, Induced plant defenses, Systemic acquired resistance, Conservation biological control

HIGHLIGHTS

Elicitors can induce plant resistance against insects and pathogens.
In maize field these elicitors also changed plant odor emissions.
The changes have minor effects on herbivores and parasitoid presence.
Elicitor treatment is compatible with biological control.

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1. Introduction

Plants attacked by arthropod herbivores respond by activating a number of defense mechanisms, including the emission of volatile organic compounds (VOCs) that attract predatory and parasitic arthropods (Dicke, 2009; Dicke et al., 1990; Turlings et al., 1990; Turlings and Wäckers, 2004). In maize, these herbivore-induced plant volatiles comprise mainly green leaf volatiles (GLVs), mono-, homo- and sesquiterpenes, as well as aromatic compounds (D'Alessandro et al., 2006; Hoballah and Turlings, 2005; Paré and Tumlinson, 1999). The emission of most herbivore-induced plant volatiles involves a number of well-understood metabolic pathways, such as the jasmonic acid (JA) pathway, the shikimic acid/tryptophan pathway, the mevalonate pathway, as well as the lipoxygenase (LOX) pathway (Paré and Tumlinson, 1999; Dudareva et al., 2004; Bruce and Pickett, 2007; Engelberth et al., 2007). Similarly, plants infected by fungi or bacteria respond with the activation of a number of defense mechanisms against these pathogens (van Loon et al., 2006; Walling, 2009), and in most cases this involves the plant hormone salicylic acid (SA) (Bari and Jones, 2009). Resistance against pathogens as well as defenses against insects can also be induced with synthetic versions of these elicitors or their derivatives (Heil and Walters, 2009; Walling, 2009). Besides increasing the direct defenses in the plants, such treatments may also enhance the volatile defense signals. For instance, spraying plants with methyl jasmonate (MeJA), a volatile derivative of JA, induces the emission of volatiles that are also induced by herbivore feeding (Degenhardt and Lincoln, 2006; Heil and Walters, 2009; Ozawa et al., 2008; Zhang et al., 2009). This can lead to increased parasitism of caterpillars as was observed by Thaler (1999) near tomato plants that she had treated with JA.

Pathogen resistance can be induced by the elicitor benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH), which mimics the effects of salicylic acid (SA) and results in a reduction of the impact of several pathogens in different plant species by inducing systemic acquired resistance (SAR) (Friedrich et al., 1996; Görlich et al., 1996), but see (Heidel and Baldwin, 2004). BTH is commercially applied in poaceous and solanaceous crops as well as sunflowers and grapevine as a preventive measure against pathogen growth (Goellner and Conrath, 2008; Perazzolli et al., 2008; Vallad and Goodman, 2004).

In many plant species there is crosstalk between the SA and the JA pathways, where the increased activity of one pathway diminishes the activity of the other (Kunkel and Brooks, 2002). Hence, the application of an elicitor to induce one of these two pathways might result in a suppression of other defense mechanisms depending on the interactions between the pathways (Beckers and Spoel, 2006; Thaler et al., 2002a,b; Walters and Heil, 2007). There are exceptions (Boughton et al., 2006), but in general, herbivorous insects perform better on plants with an activated SA pathway (Rayapuram and Baldwin, 2007; Smith et al., 2009; Taylor et al., 2004; Thaler et al., 2002a,b). However, this may be different if indirect defense signals and tritrophic interactions are also taken into account. In fact, a recent study (Rostás and Turlings, 2008) shows that treatment with BTH not only increases resistance to the pathogenic fungus *Setosphaeria turcica* in maize, but also strongly enhances the attractiveness to the parasitoid *Microplitis rufiventris* (Kokujev) (Hymenoptera: Braconidae) if the treated plants are subsequently infested with hosts of the parasitoid, the larvae of *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae). Ongoing experiments indicate that other parasitoid species also show increased attraction to BTH-treated maize plants (I. Sobhy, personal communication).

Hence, induction of pathogen resistance may affect the emission of volatile compounds, and thereby indirectly enhance resistance against herbivores via the third trophic level. The aim of the current study was to assess whether treating maize plants with BTH or MeJA

affects the plants' direct and indirect defenses against important pest species under realistic field conditions. Direct effects can be through the induced production of defense compounds or volatiles that repel the pests, whereas indirect effects would be the enhanced attraction of natural enemies of herbivores. To study this, four experiments were conducted at different time-points over the year in maize fields in the subtropical lowlands of Mexico to determine the effects of elicitors on volatile emission of maize plants, herbivory, parasitism, and plant performance.

2. Material and methods

2.1. Maize fields

Seven field experiments with *Zea mays* (cv Tuxpeño Sequía) planted on six dates were conducted at the International Wheat and Maize Improvement Center (CIMMYT) experimental station near Agua Fría, Puebla State, Mexico (20°26'56.93"N, 97°38'23.98"W, 98 masl). Two fields were planted in summer 2008 (replicate 1 on 9 June 2008 and replicate 2 on 16 June 2008) and four fields in winter 2009 (replicate 3 on 6 February 2009, replicate 4 on 9 February 2009, replicate 5 on 11 February 2009 and replicate 6 on 13 February 2009). A seventh field was planted on 6 February 2009 to evaluate the treatment effects on disease resistance and kernel production. The fields were planted in 31 rows of 25 m, with 20 cm distance between plants within the rows and 70 cm distance between rows (Fig. 1). They were regularly irrigated with sprinklers 2–4 days after sowing, and with occasional flooding from V2 developmental stage (collar of the second leaf visible) onward (Çakir, 2004). Neither seeds nor plants were treated with insecticides until the end of the experimental period. Each field was divided into plots that were used for the different treatments.

2.2. Treatment of plants with elicitors

Four types of treatments (two elicitors and corresponding control sprays) were applied to plots of four meters length and four rows wide that were evenly distributed in the field. One meter on each

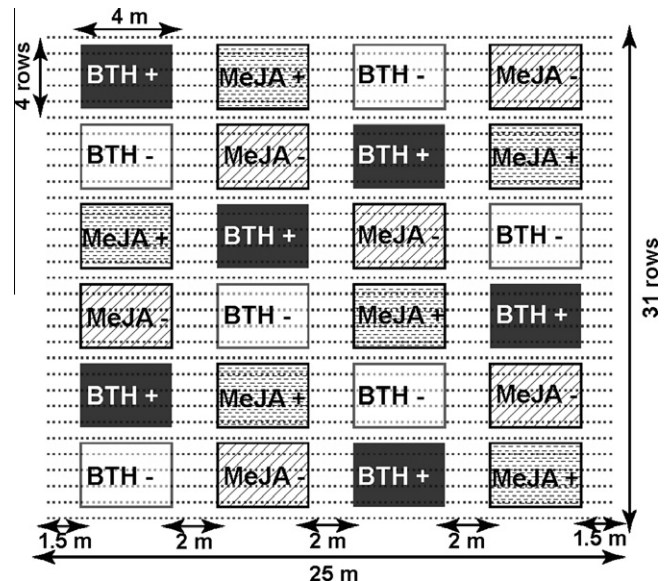


Fig. 1. Design of the field experiments. The field was 25 m × 21.5 m (31 rows 0.7 m) wide. Each plot was 4 m × 4 rows, represented by a square. Dots represent the maize plants. On each side of the field, 1.5 m was left untreated. Between plots, 2 m and 1 row was left untreated. The treatments (BTH+, BTH-, MeJA+ and MeJA-) were applied to young maize seedlings in evenly distributed plots. This treatment was applied on different fields on six occasions throughout the year.

side of the rows and one row between plots were left untreated, as buffer zones (see Fig. 1). For one of the treatments, plants were sprayed with 0.3 mM benzo (1, 2, 3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) (Bion[®], Syngenta Crop Protection, Pratteln, Switzerland) at V2–V3 developmental stage of the seedlings with 2–3 leaves present after the cotyledon (treatment BTH+). The control (treatment BTH–) consisted of a similar treatment, but contained only wetting powder at 0.3 mM without the active ingredient BTH (Bion 50WG, Syngenta Crop Protection, Basel, Switzerland). For the second elicitor treatment, plants were sprayed with 5 mM methyl jasmonate in 0.5% ethanol solution (treatment MeJA+) or with 0.5% ethanol solution (treatment MeJA–) as a control treatment. The applied dosages were based on those used in previous studies (Boughton et al., 2005; Boughton et al., 2006) and initial laboratory studies confirmed their effectiveness (data not shown). Before the treatments, herbivore damage and presence of *Diabrotica* spp. (Coleoptera: Chrysomelidae), *Spodoptera frugiperda* (Lepidoptera: Noctuidae), aphids (Homoptera: Aphididae), as well as pathogen presence were checked for all plots.

2.3. Volatile collection and analysis (Experiment 1)

Volatiles were sampled three to four days after elicitor application. For this, plants were covered with a Nalophan sleeve (Omya AG, Oftringen, Switzerland) 150 mm diameter, 50 cm length. At the bottom, the sleeve was closed with a plastic seal below the oldest leaf and at the top it was attached to a metal wire, to prevent mechanical damage due to wind moving the sleeve. A tubular glass device (23 × 17 × 12 mm) with an open screw cap was attached to the bottom of the bag in order to insert a SuperQ filter (Analytical Research Systems, Inc., Gainesville FL, USA). Air was pulled through the filter tube with the use of an air-sampling pump (SKC 222 series, Blanc Labo S.A., Lonay, Switzerland) for 3 h at 0.6 L/min. For each of the 12 replications, volatiles were collected simultaneously from a treated plant (BTH+ or MeJA+) and its respective control (BTH– or MeJA–). The volatiles were then eluted from the filters and analyzed in GCMS as described by D'Alessandro and Turlings (2005).

2.4. Herbivory and parasitism (Experiment 2)

Seven days after elicitor treatment, six to ten plants in each plot were rated for herbivore damage to the shoot, which could be assigned to different species. The feeding damage by *S. frugiperda* was rated on a scale from 0 to 5: 0 = absence of damage, 1 = very slight damage, 2 = slight damage, 3 = spread damage, 4 = severe damage and 5 = almost complete removal of all foliar tissue, based on other leaf damage studies (Kumar, 2002; Wiseman et al., 1966). A similar scale was used to assess the damage by adult *Diabrotica* spp. beetles. In addition, while scoring the damage, the number of insect herbivores on each plant was counted. The herbivores that were observed included *Diabrotica* spp. adults, flea beetles (Coleoptera: Chrysomelidae: Alticini), aphids, leafhoppers (Hemiptera: Cicadellidae) and planthoppers (Hemiptera: Fulgoromorpha). Ten to thirteen days after treatment all plants in the plot were cut off to count and collect the caterpillars feeding on them. The only two species found were *S. frugiperda* and *Diatraea saccharalis* (Fabricius) (Lepidoptera: Pyralidae). The number of plants that were infested by either of these species or by both was recorded. Subsequently all caterpillars were placed individually in single compartments of 24-well ELISA plates containing artificial maize-based diet (Hoballah et al., 2004) and reared at the CIMMYT's entomological facility in Texcoco de Mora, Mexico State, Mexico, under controlled conditions (27 ± 2 °C and 55 ± 5% R.H.) until adult emergence or, in the case of parasitism, emergence of parasitoids (Hoballah et al., 2004). The adult parasitoids as well as dead parasitoid larvae and cocoons were individually conserved in 90% eth-

anol in centrifuge tubes. Adult parasitoids were identified under a binocular with the help of a manual (Cave, 1995). Dead larvae and pupae of parasitoids were identified based on the adults that emerged from identical larvae and pupae.

2.5. Potted plant experiment (Experiment 3)

Maize plants (cv Tuxpeño Sequía) were sown on 16 June 2008, individually in black 10 l PVC pots in an insect-proof green house, located next to the fields at the experimental field station. Fifteen maize plants per treatment were sprayed with the elicitors (MeJA+ and BTH+) and their respective controls (BTH– and MeJA–), at the V3–V4 (three to four leaves after cotyledon) developmental stage. After 24 h, potted maize plants were placed in a maize field with plants at a similar growth stage in groups of four with each treatment. To exclude any position effect, the position of each treatment was rotated between the groups. Groups were evenly distributed over the field with a distance of five meters between groups. Twenty days later, pathogen infestation and herbivore damage were rated. Pathogen infestation was assessed by counting the number of necroses on the most recent fully developed leaf. To identify the pathogen a sample of necrotic spots was study under a microscope. In this experiment, herbivory damage was assessed on a scale based on Wiseman et al. (1966). Feeding by *Diabrotica* spp. adults was rated using a scale from 0 to 10 (0 = no visible damage, 1 = few pinhole-type injuries, 2 = several small pinholes, 3 = small amount of shot-hole type injury with few lesions, 4 = several shot-hole type injuries and few lesions, 5 = several lesions, 6 = several lesions, portions of plant eaten away, 7 = several lesions, portions of plant eaten away, few areas dying, 8 = several portions eaten away and areas dying, 9 = most of the leaves eaten away and more areas dying, 10 = plant dead or dying). Flea beetle damage (whitish stripes on leaves) was rated using the same scale. The *S. frugiperda* and *D. saccharalis* caterpillars that were retrieved from the plants were also counted. After pathogen and herbivory assessment, the plants were cut, placed individually in paper bags, heated in an oven at 80 °C for 24 ± 1 h to measure dry weight.

2.6. Pathogen disease resistance and plant fitness (Experiment 4)

One field (25 m × 30 rows) was planted and treated with the elicitors as described above (2.2). No insects were collected from these plants, and the plants were left in the field until maturity to assess kernel production and plant performance parameters. Seven days before harvest, pathogen infestation and disease symptoms were rated. The infection of the plants by the fungal pathogen *Bipolaris maydis* (Y. Nisik. and C. Miyake), which was the major pathogen found in the fields, was rated visually using a scale from 0 to 5: 0 = no spread, 1 = slight spread, 2 = clearly notable spread, 3 = wide spread, 4 = almost complete spread, 5 = heavy spread as described by Sharma and Payak (1990). In addition, lodging (breakage of the stalk or root system) and stunting of the plants was rated on a similar scale. At harvest, the number of cobs was recorded. Furthermore, we measured the weight of the harvested cobs and estimated the cob water content by subtracting dry from fresh weight.

2.7. Statistical analysis

2.7.1. Volatile emission

Data of the volatile emissions were tested for homogeneity of variances (Levene test) and analyzed using a pair-wise comparison (*t*-test). Each treatment was compared only with its respective control (i.e. BTH+ vs BTH– and MeJA+ vs MeJA–), also because the volatile emissions of the two treatments were measured on different days, i.e. under different conditions. When the variances

were not homogenous, the data were analyzed using the Wilcoxon pairwise comparison.

2.7.2. Herbivory and parasitism

When comparing the number of herbivores, data were analyzed using analysis of variance (ANOVA) on the same factors. The data were analyzed using the Shapiro–Wilk test of normality. If the dataset was not normally distributed, it was log-and square root-transformed. In cases where transformation did not improve the data, they were tested using the Kruskal–Wallis rank sum test, with the four treatments BTH+, BTH–, MeJA+ and MeJA– as explanatory factors. Herbivore damage rating data were analyzed using ANOVA. The data were tested for normality with the Shapiro–Wilk test of normality. If the data were not normally distributed and data transformation did not improve normality, the data were analyzed using the Kruskal–Wallis rank sum test. Data of herbivore-infested plants was analyzed using a generalized linear model (GLM) with binomial distribution, where plants were either infested or not. However, overdispersion of the data required adapting the statistical method, as suggested by Verdon et al. (2007). The difference between time-points was also tested using the quasi-binomial testing method. The parasitism data were analyzed in a similar way as for the herbivore presence data.

2.7.3. Disease rating

Estimates of disease spread were tested using ANOVA with the four treatments BTH+, BTH–, MeJA+ and MeJA– as explanatory factors. The data were tested for normality using the Shapiro–Wilk test of normality. In cases where the data were not normally distributed, the data were tested using the Kruskal–Wallis rank sum test.

3. Results

3.1. Volatile emission (Experiment 1)

Maize plants in the field emitted several well-known herbivore-induced VOCs, including GLVs, such as (Z)-3-hexenyl acetate, aromatic compounds, such as phenethyl acetate and methyl salicylate, as well as a series of mono-, homo- and sesquiterpenes. Consistent with a previous laboratory study (Rostás and Turlings, 2008), plants treated with BTH (BTH+) tended to emit less homoterpenes, sesquiterpenes and aromatic compounds than control-treated plants (BTH–), but the differences in the amount of individual compounds were not statistically significant (Table 1). By contrast, plants treated with MeJA (MeJA+) emitted higher amounts of most inducible compounds than the control-treated plants (MeJA–). This effect was statistically significant for (*E*)- β -bergamotene ($P < 0.047$) and (*E*)- α -farnesene ($P < 0.043$) (Table 1).

3.2. Herbivory (Experiment 2)

Before the treatment with elicitors, 22–25% of the plants were infested in all plots. Ten days after treatment, the number of infested plants did not differ between the treatments in the six replicates, with an average of 42.3% plants (Fig. 2). This similar infestation rate was also reflected in an equal damage by the larvae (0.40–0.58) ($F_{3,92} = 0.23$, $P < 0.874$) and in the number of *S. frugiperda* found per sampled plant (0.75–0.82) ($F_{3,139} = 0.10$, $P < 0.962$) (Table 2). However, there was a significant difference in the infestation rate for the different replicates sampled at different time-points during the year ($F_{5,137} = 18.24$, $P < 0.001$) (Fig. 2).

Other herbivores were also found in similar abundance in all treatments (Table 2). *Diabrotica* spp. damage ($F_{3,116} = 0.08$, $P < 0.968$) as well as the number of *Diabrotica* beetles ($F_{3,92} =$

0.58, $P < 0.627$) was similar across treatments. The same was true for the presence of aphids ($F_{3,92} = 0.18$, $P < 0.949$). Overall, summing the six replicates, we collected almost equal numbers of *S. frugiperda* caterpillars (1420 in BTH+, 1441 in BTH–, 1379 in MeJA+, and 1388 in MeJA–), as well as *D. saccharalis* (309 in BTH+, 345 in BTH–, 327 in MeJA+, and 264 in MeJA–). *D. saccharalis* was found only in summer 2008.

3.3. Parasitism rates and parasitoids (Experiment 2)

In replicate 3, parasitism by *Chelonus insularis* was significantly higher in BTH+ treated plants ($14.8 \pm 4.48\%$) compared to BTH– ($6.51 \pm 2.42\%$) treated plants (GLM, $P < 0.014$). In this replicate, there was also a trend of increased parasitism by *C. insularis* in MeJA+ treated plants ($7.05 \pm 1.35\%$), compared with MeJA– treated ($10.20 \pm 4.73\%$) plants ($P < 0.051$). However, in the other replicates, there were no significant differences between the treatments for any of the wasp species that were retrieved. When cumulating all parasitoid species, there was a trend that parasitism was higher in BTH+ treated plots than in BTH– treated plots ($P < 0.065$) (Fig. 3). Particularly, in replicate 2 there was a slightly higher parasitism in BTH+ plots than in BTH– ($P < 0.106$), but this trend was reversed in replicate 6, with more parasitism in BTH– plots ($P < 0.022$). The parasitism rates did not differ between MeJA+ and MeJA– ($P > 0.05$ for all replicates).

The main parasitoid during winter and summer was the egg-larval parasitoid *insularis* with almost 20% parasitism rate of *S. frugiperda* (Table 3). The second most frequent parasitoid was *Eiphosoma vitticolle*, followed by *Pristomerus spinator* and *Campoletis sonorensis*. The next most frequent parasitoid was the braconid *C. marginiventris*, which was also found both in winter and in summer. *Aleiodes laphygmae* and *C. cautus*, as well as *Ophion flavidus* were found in small numbers with only one occurrence for the latter. For instance, the specialist parasitoid *A. diatraeae* was only reared from *D. saccharalis* larvae during the summer experiments. Tachinidae were also found only in summer, as well as *A. laphygmae* and *O. flavidus*. On the other hand, *P. spinator* and *C. sonorensis* were found only in winter. The egg-larval parasitoid *C. cautus* was found only in winter 2009, and *E. vitticolle* was also found mostly in the winter.

3.4. Potted plant experiment (Experiment 3)

The effect of the elicitors was also tested in a more standardized field experiment with potted plants that were divided into groups of equal mean plant size and treated in the greenhouse before transfer to the field. Twenty days after treatment, BTH+ plants weighed significantly more than the plants of the other treatments ($F_{3,56} = 8.26$, $P < 0.001$) (Table 4). As expected, we observed a significantly lower number of necrosis on BTH+ plants compared to BTH–, MeJA+ and MeJA– plants ($F_{3,56} = 8.53$, $P < 0.007$) (Fig. 4). Visual inspection under the microscope revealed that southern leaf blight (*B. maydis*) was identified as the pathogen causing the necrosis spots. Interestingly, the BTH– treated plants had increased numbers of necrosis, compared to MeJA+ and MeJA–. Flea beetle damage ($F_{3,56} = 0.72$, $P < 0.542$) and *Diabrotica* spp. damage ($F_{3,56} = 0.45$, $P < 0.721$) did not differ between the four treatments. Only few caterpillars were feeding on these plants and therefore no statistical test was applied.

3.5. Pathogen disease resistance and plant fitness (Experiment 4)

In the fourth experiment we grew plants in the fields and treated them with elicitors similarly as in experiment 2. Seven days before harvesting all treatments showed similar *B. maydis* symptoms, the main fungal pathogen found in the field (Table 5)

Table 1

Mean amount of VOCs (\pm SEM) emitted by maize plants treated with BTH+ ($n = 12$), BTH- ($n = 10$), MeJA+ ($n = 11$) and MeJA- ($n = 12$) in the field. Individual compounds are summed into major chemical groups of volatiles and their mean is indicated in bold. Data were analyzed using pairwise Student's *t*-test. Significant differences and trends are in bold and an asterisk indicates significant difference ($P < 0.05$), a large dot indicates a statistical trend ($0.05 < P > 0.10$).

| Volatile compounds (ng \pm SEM) | BTH+ | BTH- | MeJA+ | MeJA- |
|--|-----------------------------------|-----------------------------------|-------------------------------------|--------------------------------------|
| (Z)-3-hexenal | 1.16 \pm 0.47 | 1.01 \pm 0.52 | 4.84 \pm 1.85 | 3.23 \pm 1.45 |
| (E)-2-hexenal | 0.00 \pm 0.00 | 0.00 \pm 0.00 | 0.00 \pm 0.00 | 5.41 \pm 5.42 |
| (Z)-3-hexen-1-ol | 4.51 \pm 3.01 | 3.04 \pm 1.32 | 13.51 \pm 6.49 | 8.89 \pm 4.04 |
| 1-butanol, 3-methyl-, acetate ^a | 2.49 \pm 0.46 | 7.95 \pm 2.89 | 3.45 \pm 0.52 | 2.58 \pm 0.60 |
| (Z)-3-hexenyl acetate ^a | 31.86 \pm 9.48 | 30.48 \pm 9.83 | 148.55 \pm 58.88 | 62.99 \pm 23.15 |
| Acetic acid hexyl ester | 2.34 \pm 0.49 | 3.23 \pm 0.79 | 5.20 \pm 1.13 | 3.83 \pm 0.50 |
| Total GLVs | 42.35 \pm 12.93 | 45.70 \pm 14.38 | 175.57 \pm 66.82 | 86.96 \pm 29.89 |
| α -pinene | 1.18 \pm 0.39 | 1.96 \pm 0.88 | 2.03 \pm 0.53 | 1.73 \pm 0.56 |
| β -myrcene | 2.88 \pm 1.16 | 4.88 \pm 2.92 | 4.49 \pm 1.16 | 4.14 \pm 1.41 |
| (Z)- β -ocimene | 12.00 \pm 4.85 | 25.92 \pm 15.62 | 16.66 \pm 11.90 | 22.29 \pm 9.69 |
| linalool | 22.68 \pm 5.66 | 19.58 \pm 6.47 | 34.47 \pm 9.09 | 27.49 \pm 9.98 |
| Total monoterpenes | 38.74 \pm 9.36 | 52.35 \pm 25.30 | 57.67 \pm 19.76 | 55.67 \pm 21.65 |
| (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) | 14.44 \pm 3.97 | 43.23 \pm 26.09 | 59.24 \pm 37.27 | 15.31 \pm 7.06 |
| (3E,7E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) | 0.39 \pm 0.39 | 4.55 \pm 2.49 | 0.31 \pm 0.25 | 0.95 \pm 0.86 |
| Total homoterpenes | 14.82 \pm 4.21 | 47.77 \pm 28.29 | 59.56 \pm 37.25 | 16.27 \pm 7.11 |
| (+)-cycloisativene | 9.55 \pm 3.42 | 10.09 \pm 4.88 | 18.42 \pm 7.07 | 9.38 \pm 5.31 |
| α -copaene | 8.53 \pm 4.02 | 17.64 \pm 12.67 | 11.20 \pm 3.82 | 4.52 \pm 2.56 |
| (E)- β -caryophyllene | 7.07 \pm 2.98 | 19.29 \pm 10.67 | 12.90 \pm 3.82 | 8.58 \pm 3.45 |
| (E)- α -bergamotene | 8.85 \pm 4.33 | 27.28 \pm 14.89 | 11.74 \pm 3.58 | 4.07 \pm 1.144* |
| (E)- β -farnesene | 5.68 \pm 3.64 | 6.56 \pm 3.91 | 8.41 \pm 3.18 | 1.74 \pm 0.57* |
| Total sesquiterpenes | 39.29 \pm 13.17 | 100.84 \pm 44.58 | 62.67 \pm 13.95 | 27.60 \pm 11.39* |
| Phenethyl acetate | 1.20 \pm 0.95 | 1.66 \pm 0.71 | 2.22 \pm 0.67 | 2.59 \pm 0.97 |
| Methyl salicylate | 2.30 \pm 0.99 | 2.75 \pm 1.02 | 1.34 \pm 0.70 | 2.07 \pm 0.81 |
| Total aromatics | 3.50 \pm 1.25 | 4.41 \pm 1.19 | 3.57 \pm 1.19 | 4.45 \pm 1.38 |

^a Data did not fit with assumptions of equal variance between groups. Hence, a non-parametric test was applied (Wilcoxon pairwise comparison).

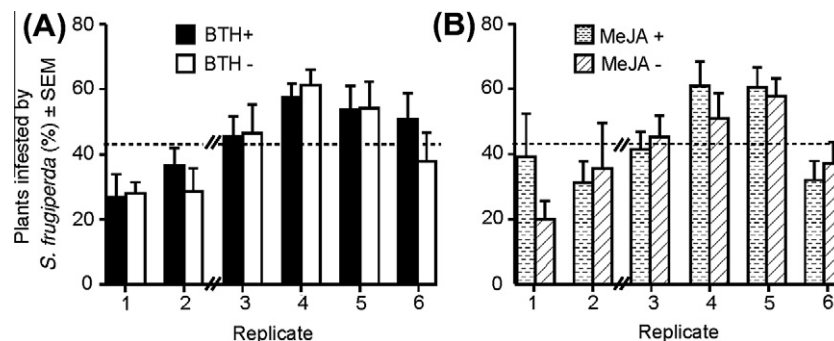


Fig. 2. Infestation of maize plants by caterpillars of *Spodoptera frugiperda* (mean percentage of infested plants \pm SEM) on (A) BTH+ and BTH- treated plants and (B) on MeJA+ and MeJA- treated plants. The dotted line indicates the average infestation rate over the six replicates and four treatments. The experiments were replicated six times over time (reps 1 and 2 in June 2008 and reps 3–6 in February 2009). Data were analyzed with the general linear model (GLM).

Table 2

Mean herbivore damage (scale: 0–5 \pm SEM) and number of herbivores (\pm SEM) per maize plant treated with BTH+, BTH-, MeJA+ and MeJA-. Data were analyzed using one-way analysis of variance (ANOVA). n.s.: no significant difference between treatments ($P > 0.05$).

| | BTH+ | BTH- | MeJA+ | MeJA- | |
|--|-----------------|-----------------|-----------------|-----------------|------|
| <i>Herbivore damage \pm SEM^a</i> | | | | | |
| <i>Diabrotica</i> spp. | 1.58 \pm 0.11 | 1.66 \pm 0.13 | 1.65 \pm 0.11 | 1.63 \pm 0.12 | n.s. |
| <i>Spodoptera frugiperda</i> | 0.54 \pm 0.12 | 0.58 \pm 0.13 | 0.40 \pm 0.11 | 0.46 \pm 0.13 | n.s. |
| <i>Number of herbivores \pm SEM</i> | | | | | |
| <i>Spodoptera frugiperda</i> ^b | 0.82 \pm 0.09 | 0.75 \pm 0.10 | 0.76 \pm 0.09 | 0.76 \pm 0.09 | n.s. |
| <i>Diatrea saccharalis</i> ^c | 0.54 \pm 0.10 | 0.61 \pm 0.09 | 1.02 \pm 0.48 | 0.45 \pm 0.07 | n.s. |
| <i>Diabrotica</i> spp. ^b | 0.27 \pm 0.05 | 0.38 \pm 0.06 | 0.35 \pm 0.09 | 0.28 \pm 0.06 | n.s. |
| Aphids ^a | 0.08 \pm 0.03 | 0.13 \pm 0.05 | 0.05 \pm 0.02 | 0.08 \pm 0.03 | n.s. |

^a Damage was measured in winter 2009 only.

^b Insect species was found both in summer 2008 and winter 2009.

^c Insect species was found only in summer 2008 field assay.

($F_{3,92} = 1.66$, $P < 0.181$), lodging ($F_{3,92} = 0.59$, $P < 0.620$), or stunting ($F_{3,92} = 1.90$, $P < 0.135$), did not show a significant difference among treatments. There was also no difference in cob number ($F_{3,92} = 0.53$, $P < 0.666$), cob weight ($F_{3,92} = 0.44$, $P < 0.730$), and kernel humidity ($F_{3,92} = 0.66$, $P < 0.578$) among treatments.

4. Discussion

In this study we investigated whether the application of two potent elicitors of pathogen resistance (BTH) and insect defense responses (MeJA) affected the herbivores and pathogens in

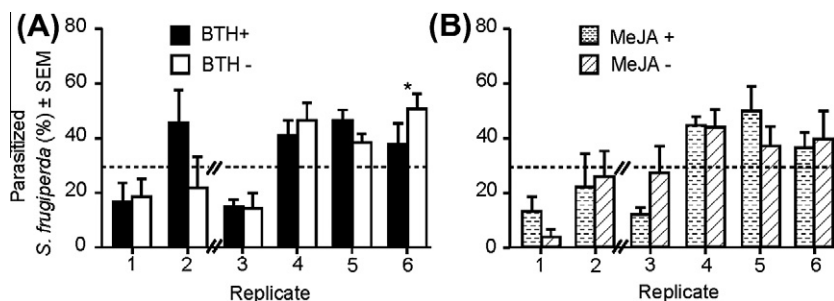


Fig. 3. Parasitism of *Spodoptera frugiperda* (% of collected larvae \pm SEM) found on plants treated with (A) BTH+ and BTH- and (B) MeJA+ and MeJA-. The dotted line indicates the average parasitism rate over the six replicates and four treatments. The experiments were replicated six times over one year (reps 1 and 2 in June 2008 and reps 3–6 in February 2009). Data were analyzed by GLM.

Table 3

Total number of parasitoids emerged from collected *S. frugiperda* larvae. The insects were collected in summer 2008 and winter 2009.

| Species | BTH+ | BTH- | MeJA+ | MeJA- | Total |
|---|------|------|-------|-------|-------|
| <i>Chelonus insularis</i> | 251 | 290 | 242 | 228 | 1011 |
| <i>Eiphosoma vitticolle</i> | 58 | 64 | 78 | 73 | 273 |
| <i>Pristomerus spinator</i> ^a | 30 | 45 | 41 | 32 | 148 |
| <i>Campoletis sonorensis</i> ^a | 9 | 26 | 14 | 15 | 64 |
| <i>Apanteles diatraeae</i> ^b | 7 | 4 | 4 | 7 | 22 |
| <i>Cotesia marginiventris</i> | 7 | 4 | 2 | 3 | 16 |
| Tachinidae ^b | 5 | 4 | 2 | 3 | 14 |
| <i>Aleiodes vaughanii</i> ^b | 2 | 1 | 1 | 1 | 5 |
| <i>Chelonus cautilus</i> ^a | 2 | 0 | 1 | 1 | 4 |
| <i>Ophion flavidus</i> ^b | 0 | 0 | 1 | 0 | 1 |

^a Insects found only in winter 2009.

^b Insects found only in summer 2008.

subtropical maize fields in Mexico. In particular, we evaluated the effect of the elicitors on parasitism of caterpillars of *S. frugiperda*, the main pest insect of maize in Mexico. Consistent with laboratory experiments, the treatment with the elicitor BTH (BTH+) slightly reduced the volatile emission by herbivore-infested maize seedlings compared to the control treatment (BTH-) (Table 1; Rostás and Turlings, 2008) and the treatment with MeJA (MeJA+) slightly increased the volatile emission of two major sesquiterpenes compared to its control treatment (MeJA-) (Degenhardt and Lincoln, 2006). It should be noted that the emissions of BTH and MeJA could not be directly compared because volatiles for these treatments were collected on different days under different conditions.

The differences in volatile emissions caused by the treatments had no apparent effect on the infestation by herbivores (Table 2 and Fig. 2) and only slightly affected the parasitism rate of *S. frugiperda* (Table 3 and Fig. 3). Hence, our field data do not exactly mirror the findings from the laboratory study by Rostás and Turlings (2008), in which BTH treatment strongly increased the attractiveness of caterpillar-damaged maize plants to a parasitoid. We also did not find an effect of MeJA treatment on herbivory or

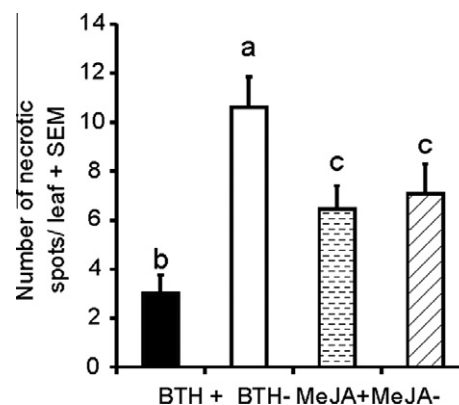


Fig. 4. Number of necrotic spots per last fully developed leaf ($n = 15$) + SEM. Data were analyzed with one-way analysis of variance (ANOVA). A different letter indicates a significant difference ($P < 0.05$). Further data of the same experiment are shown in Table 4.

parasitism, despite the fact that this treatment increased the emission of sesquiterpenes. Halitschke et al. (2000) also found that application of MeJA on wild tobacco increases the emission of sesquiterpenes and, in their case, this resulted in increased predation of hornworm eggs (Kessler and Baldwin, 2001). Similarly, Thaler (1999) had found that treatment of tomato plants with jasmonic acid increases parasitism in the field. Different application methods that were used in these studies may explain the differential effects on insect presence. In our case, spraying with MeJA must have activated the plant defenses, but the MeJA probably evaporated soon after application, while in the case of Halitschke et al. (2000) and Kessler and Baldwin (2001) MeJA was applied on the plants in lanolin paste, resulting in a slow release and prolonged exposure of the plants and possible direct effects of MeJA on the recruitment of insects.

Table 4

Mean plant dry weight ($g \pm$ SEM), herbivore damage (scale 0–10 \pm SEM) and number of herbivores (\pm SEM) per maize plant treated with BTH+, BTH-, MeJA+ and MeJA-. $n = 15$ for all treatment. Data were analyzed with one-way analysis of variance (ANOVA) and Tukey's post hoc test. A different letter in the same row indicates a significant difference at $P < 0.05$.

| | BTH+ | BTH- | MeJA+ | MeJA- |
|--|------------------|------------------|------------------|------------------|
| Plant dry weight ($g \pm$ SEM) | 5.70 \pm 0.35a | 4.13 \pm 0.25b | 4.60 \pm 0.22b | 4.13 \pm 0.17b |
| <i>Herbivore damage \pm SEM</i> | | | | |
| <i>Diabrotica</i> spp. | 0.80 \pm 0.22a | 1.07 \pm 0.21a | 0.80 \pm 0.26a | 1.07 \pm 0.23a |
| Flea beetle | 3.53 \pm 0.59a | 3.53 \pm 0.53a | 4.60 \pm 0.61a | 4.33 \pm 0.81a |
| <i>Number of herbivores</i> | | | | |
| <i>Diatrea saccharalis</i> | 4 | 4 | 2 | 5 |
| <i>Spodoptera frugiperda</i> | 0 | 1 | 1 | 2 |

Table 5

Disease symptoms caused by *Bipolaris maydis* (scale: 0–5) and several fitness measures of maize plants treated with BTH+, BTH–, MeJA+ and MeJA– at the date of harvesting. Data were analyzed with one-way analysis of variance (ANOVA).

| | BTH+ | BTH– | MeJA+ | MeJA– | P |
|---------------------------------|--------------|--------------|--------------|--------------|--------|
| <i>Disease symptoms (± SEM)</i> | | | | | |
| Necrosis | 2.08 ± 0.57 | 1.58 ± 0.33 | 1.21 ± 0.27 | 1.42 ± 0.42 | <0.181 |
| Lodging | 8.29 ± 0.43 | 8.54 ± 0.63 | 7.63 ± 1.21 | 7.67 ± 0.63 | <0.621 |
| Stunting | 4.96 ± 0.49 | 4.25 ± 0.20 | 4.42 ± 0.29 | 3.67 ± 0.25 | <0.135 |
| <i>Fitness measures ± SEM</i> | | | | | |
| Cobs/plant | 0.92 ± 0.05 | 0.97 ± 0.04 | 0.94 ± 0.03 | 0.96 ± 0.03 | <0.666 |
| Cob weight (g) | 1210 ± 130 | 1330 ± 150 | 1310 ± 140 | 1260 ± 140 | <0.728 |
| Grain humidity (%) | 38.56 ± 0.76 | 38.33 ± 1.10 | 37.55 ± 1.03 | 36.73 ± 1.36 | <0.578 |

The experiments were replicated six times, covering one summer and one winter season. We feel that this relatively high number of replications is one of the strong points of the study, but it resulted in obvious confounding effects that could have masked treatment effects. Not only abiotic, but also biotic conditions were considerably different for the two seasons, which is most clearly reflected in substantial differences in the species compositions of herbivores and parasitoids (Tables 2 and 3). Several field-related factors might also explain why the treatments had little effect on the responsiveness of the herbivores and parasitoids in our specific field trials. First of all, the application of elicitors may have occurred after most plants were already induced by the natural infestation with herbivores and pathogens. These early antagonists are likely to have triggered SA and JA-mediated defenses prior to elicitor application. Indeed, we observed flea beetles and leaf bugs on the maize plants already during the first days after the emergence of the cotyledon. These insects have been shown to induce the emission of similar amounts of volatiles in wild tobacco (Kessler and Baldwin, 2001). Moreover, many of the maize plants were damaged by adult *Diabrotica* beetles prior to application of elicitors.

In contrast to the sesquiterpenes, some volatile compounds that are affected by elicitor treatments in the laboratory (Rostás and Turlings, 2008), such as indole, were not emitted under the field conditions. This might have been due to the different herbivores feeding on the plants in the field and/or to additional biotic and abiotic stresses that could have altered the volatile emission in the field. Various biotic and abiotic factors are known to strongly influence the quantity and quality of volatile compounds emitted by plants (Gouinguéné and Turlings, 2002; Loreto and Schnitzler, 2010; Takabayashi et al., 1994). Although the fields were irrigated at regular intervals, high temperatures were measured throughout the experimental period, possibly causing heat stress. In the study by Gouinguéné and Turlings (2002), maize plants emitted increased amounts of homoterpenes and sesquiterpenes at 37 °C. These temperatures were reached on several days of the experimental period. Furthermore, these temperatures regularly dehydrated the top portion of the soil where the plants were growing, which in early stages can have caused drought stress, which in turn can affect VOC emission (Gouinguéné and Turlings, 2002; Peñuelas and Staudt, 2010).

The maize plants were also subjected to a number of varying biotic factors that affect the VOC emissions and probably the presence and parasitism of *S. frugiperda*. For instance, the collected *S. frugiperda* larvae ranged from first to fourth instar and size differences likely caused variation in VOC emissions and parasitism (Gouinguéné et al., 2003). Also, various other herbivores were observed on the shoots of maize seedlings, such as *Diabrotica* beetles, aphids, flea beetles, plant hoppers and leafhoppers, as well as thrips. The presence of these herbivores varied between summer and winter (Table 2) and considerable differences among herbivores in feeding habits likely resulted in differential volatile emissions and thereby the attraction of parasitoids (Turlings et al., 1998a,b). All these herbivores also feed on the plants as adults

and, unlike larval herbivores, can rapidly colonize a field without oviposition having to occur first on the plants. Indeed, they were observed on the plants shortly after the emergence of the seedlings and likely induced defense responses before elicitor treatments, thereby affecting the outcome of the study.

Predictions about how changes in VOCs emissions may affect parasitoid attraction are virtually impossible because very little is known about which specific compounds attract parasitoids. Many parasitoids are attracted to host-induced maize VOCs and their responses are often flexible and increase for a specific odor if they encounter hosts while perceiving that odor (Turlings et al., 1993; Vet et al., 1995; Tamò et al., 2006). This is also true for specific compounds within a blend. For instance, Schnee et al. (2006) found that a typical blend of inducible maize sesquiterpenes released from *Arabidopsis* plants that were transformed with a maize terpene-synthase gene, was only attractive to the parasitoid *C. marginiventris* after female wasps had experienced the sesquiterpenes during an encounter with hosts. Similarly, the aromatic compound indole, another important volatile in the maize blend only contributes to attraction of *C. marginiventris* after such a positive association with hosts (D'Alessandro et al., 2006). This effect of associative learning is common among parasitoids (Turlings et al., 1993; Vet et al., 1995) and allows great adaptability to new cues. This might also allow them to adapt to changes caused by elicitor treatments. The compounds that are essential for innate attraction of parasitoids of *S. frugiperda* still need to be determined. These elusive key compounds may be released only in very small amounts (D'Alessandro et al., 2009) and therefore not detectable in our volatile collections. The effect of the elicitors on the emissions of these key attractants remains to be determined.

The fact that a change in VOC emissions did not translate into changes in parasitism could also be explained by the large variety of parasitoid species each with its own strategy to locate *S. frugiperda* larvae. The parasitoid species composition varied strongly with the season and several species occurred only during the winter or only during the summer (Table 3). The main parasitoid, *C. insularis* was dominant during the winter as well as the summer, representing more than 50% of the wasps collected. This species has also been found in high numbers in previous Mexican field studies (Molina-Ochoa et al., 2003). *C. insularis* is an egg-larval parasitoid, which means that it oviposits in the egg stage of its hosts and then further develops inside the larval stage (Jourdie et al., 2009; Wheeler et al., 1989). Parasitism by *C. insularis* was higher in BTH-treated plots only in one out of six replicates. Various studies have found that certain egg parasitoids are attracted to VOCs emitted by plants upon egg deposition (Hilker and Meiners, 2006; Moraes et al., 2009). If this is also the case for *C. insularis*, this attraction was not consistently affected by the elicitor treatments (Table 3).

For the other parasitoid species one noteworthy tendency was that fewer *C. sonorensis* wasps were found on BTH-treated plants. Although it was not statistically significant, the threefold lower occurrence on BTH-treated plants suggests a negative effect of

the treatment. In the case of *C. sonorensis*, reduced parasitism in BTH+ plots could be explained by the overall reduction in volatile emissions. Indeed, in laboratory studies, *C. sonorensis* show strongest attraction to plants that emit the largest amounts of VOCs (E. de Lange, unpubl. data).

Previous field studies have shown that treatment with BTH can increase the resistance of tomato plants to herbivores and pathogens (Inbar et al., 2001), and treatment of tomato plants with jasmonic acid increases the plant's attractiveness to parasitoids (Thaler, 1999). In our experiments with potted maize plants it was confirmed that treatment with BTH can decrease colonization of plant tissues by pathogens, but this was not evident from the other field assays and overall the treatment with BTH or MeJA only slightly affected parasitism. It is possible that under the severe conditions at the field sites, pathogens, as well as insects, may have challenged the seedlings before treatment, thereby already activating resistance responses and thus diminishing the effects of subsequent treatments. Clearer effects on insects might be obtained by treating the seeds with BTH before planting, which also induces the desired resistance to pathogens (Danielson, 2003). Furthermore, the very frequent observation of adults of *Diabrotica* spp. further suggests that root-feeding larvae of these beetles may have been present belowground. Recently it was shown that root herbivory by *Diabrotica* significantly affects resistance against herbivores and pathogens in the shoot (Erb et al., 2008, 2009).

Some studies have found no effect of BTH treatment on plant performance (Heil, 2002; Walters and Heil, 2007; Walters and Boyle, 2005). In the potted plant experiments we found that enhanced resistance to pathogens after BTH treatment was reflected in an increased biomass of 21-day old plants. This improved performance of the plants was no longer measurable at a later stage of development, possibly again because of the high and diverse disease and herbivore pressure that these plants were subjected to throughout the season. Overall, treatment with BTH had small positive effects on plant resistance and performance and no measurable negative consequences.

In conclusion, application of either BTH or MeJA on maize in subtropical field conditions did not increase parasitism of *S. frugiperda* larvae. However, based on previous findings from laboratory assays (Rostás and Turlings, 2008), as well as on the experiment with the potted plants it appears that under more moderate environmental conditions and with early treatment of the plants, BTH treatment could lead to enhanced resistance against pathogens. Here it is shown that BTH application has no negative effect on parasitism of the primary maize pest in Mexico, *S. frugiperda* and is therefore compatible with biological control in the study system. If under more moderate conditions elicitors can even be used to enhance the attraction of key larval parasitoids remains to be determined.

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