The effects of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one on two species of *Spodoptera* and the growth of *Setosphaeria turcica* in vitro

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Abstract Maize seedlings contain high amounts of glucosidically bound 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA). The effects of DIMBOA on the feeding behaviour and performance of two noctuids, *Spodoptera exigua* Hübner and *S. frugiperda* Smith, were compared. The question was raised whether *S. frugiperda*, preferring maize and other Poaceae, is better adapted to DIMBOA than *S. exigua*. In addition, the effects of DIMBOA on the mycelial growth of the plant pathogen *Setosphaeria turcica* Leonard et Suggs (causal agent of northern corn leaf blight) was assessed *in vitro*. DIMBOA had an antifeedant effect on *S. exigua* but stimulated feeding in *S. frugiperda* in dual-choice experiments. In a no-choice setup, larvae of *S. exigua* gained less biomass and had a prolonged development when feeding on an artificial diet containing DIMBOA. However, pupal weight was not significantly different between treatments. In contrast, larvae of *S. frugiperda* were not affected by DIMBOA. Strong detrimental effects of DIMBOA were found on the mycelial growth of the pathogen *S. turcica*.

Keywords DIMBOA · performance · *Setosphaeria turcica* · *Spodoptera exigua* · *Spodoptera frugiperda* · *Zea mays*

Introduction

In a number of cereals, benzoxazinones (Bx) are the major secondary metabolites and have been shown to confer resistance against herbivorous insects and pathogens. Bx occur e.g. in maize, wheat and rye but are absent in barley, oats and rice (Niemeyer and Perez 1995). Maize contains mainly DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) which is stored in the vacuole as the D-glucoside (Niemeyer 1988). Upon tissue disruption the glucoside is hydrolyzed by β-glucosidase, yielding the more toxic aglycone DIMBOA (Oikawa et al. 1999). The highest concentrations of DIMBOA can be found in seedlings or in the younger parts of a plant. As the plant matures, the levels of DIMBOA and other Bx decline rapidly (Cambier et al. 2000). However, insect feeding, artificial damage and pathogen infection may induce the accumulation of Bx (Gutierrez et al. 1988; Oikawa et al. 2004).

Numerous studies suggest that DIMBOA is a feeding deterrent and/or toxic to herbivorous insects. Adverse effects to various degrees have been shown mainly for aphids (Niemeyer and Perez 1995; Givovich and Niemeyer 1995; Escobar et al.

1999) but also for some lepidopteran maize pests such as *Ostrinia nubilalis* (Campos et al. 1989), *O. furnacalis* (Yan et al. 1999), and *Sesamia nonagrioides* (Ortego et al. 1998). Maize inbred lines with high levels of DIMBOA have been developed to counteract *O. nubilalis* (Lynch 1980). However, investigations on the effects of DIMBOA on the two maize damaging noctuid species *Spodoptera exigua* and *S. frugiperda* have not been undertaken, so far. Caterpillars of *Spodoptera exigua* and *S. frugiperda* (both Lepidoptera, Noctuidae) are serious agricultural pests. Originating from southern Asia, *S. exigua* now occurs world-wide, while *S. frugiperda* is a species confined to the New World. Both noctuids are generalists, feeding on a large variety of crop plants with an overlap in host ranges. Nevertheless, *S. frugiperda* differs from *S. exigua* as it clearly prefers members of the Poaceae, if available. Maize plants are attacked by both noctuid species but *S. frugiperda* plays a more prominent role as an economic pest of this field crop (Sparks 1979; Ashley et al. 1989; Pingali 2001; Showler 2001).

The role of DIMBOA in pathogen resistance has also been demonstrated but the evidence is often correlational (Weibull and Niemeyer 1995). Early reports positively related DIMBOA content in different maize inbred lines to resistance against *Setosphaeria turcica* (anamorph: *Exserohilum turcicum*, syn.: *Helminthosporium turcicum*), the causal agent of northern corn leaf blight (Couture et al. 1971; Toldi 1984). This fungus is specialized on maize and a number of *Sorghum* species where it causes leaf lesions. It occurs world-wide and is most damaging in humid and warm years when it may become epidemic as e.g. in the USA in 1992. In spite of the good correlation between DIMBOA content and disease resistance evidence for a direct effect of DIMBOA on the hyphae of this fungus is so far lacking.

The present study assessed the effects of DIMBOA on the feeding behaviour and performance of *S. exigua* and *S. frugiperda*. It was hypothesized that among both generalists, *S. frugiperda* is better adapted to DIMBOA than *S. exigua* as this species occurs preferentially on maize and other grasses. Furthermore, the effect of DIMBOA on the mycelial growth of *Setosphaeria turcica* (anamorph: *Exserohilum turcicum* = *Helminthosporium turcicum*) was evaluated *in vitro*.

Material and methods

General

Egg batches of *S. exigua* and *S. frugiperda* were obtained from Bayer CropScience (Monheim, Germany). After hatching, caterpillars were reared in transparent plastic boxes on artificial diet based on kidney bean-meal and agar (modified from Burton 1969) for 5 d until the L_2 stage was reached. The insects were kept in a climate chamber with a photophase of 15 h, 28 ± 1°C, 70 ± 5 % r.h. and a scotophase of 9 h, 25 ± 1°C, 95 ± 5 % r.h..

Setosphaeria turcica was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and cultivated on V8-Agar in darkness at room temperature.

Maize (*Zea mays* var. Lambada) and barley (*Hordeum vulgare* var. Bonus) were grown in the greenhouse in trays or pots, respectively, filled with standard potting soil (Einheitserde Typ P). Ambient day light was supplemented with light from 400 W sodium vapour lamps for 14 h. The temperature range was 20-27°C.

DIMBOA extraction

DIMBOA was isolated following the method described by Hartenstein et al. (1992). Maize seedlings were harvested 10 d after sowing by cutting the plants above the soil level. Seedlings

were weighed, cut into pieces of about 4 cm length and frozen at -20°C until required. For extraction 500 g of frozen plant material was left to thaw at room temperature for 90 min. The shoots were homogenized in 1000 ml ethyl acetate with a hand-held mixer (ESGE, Switzerland) and normal suction filtration was applied to separate plant tissue from slurry. Using a separatory funnel, the aqueous layer was separated from the organic layer and discarded. The organic layer was extracted with saturated $NaHCO_3$ solution (8 x 50 ml) and acidified to *pH* 2. The aqueous solution was re-extracted with ethyl acetate (5 x 50 ml) and the combined extracts were dried over Na_2SO_4. The extract was filtered through filter paper and evaporated to dryness *in vacuo*. Diethyl ether (20 ml) was added to the residue. The suspension containing crystalline DIMBOA was shaken carefully and filtered through a glass frit. The identity of the extracted DIMBOA was confirmed by TLC and melting point determination. For TLC, DIMBOA was dissolved in ethyl acetate. An authenticated sample of DIMBOA was used as standard for co-chromatography. TLC was performed on a silica gel plate (1 mm thickness, F_{254}; Merck, Darmstadt, Germany) using ethyl acetate:toluol 2:1 (+ 1% v/v acetic acid) as the mobile phase. For detection, the plate was sprayed with methanolic $FeCl_3$ solution (2% w/v), which reacted with DIMBOA to form a blue complex. After re-crystallization from methanol the melting point (168-169°C) was confirmed with a Kofler microscope. Four extraction procedures were carried out in total to obtain sufficient amounts of DIMBOA for the bioassays.

Feeding preference

Barley leaves (3rd leaf of 14 d old plants), which do not contain Bx, were cut into pieces of 5 cm length. DIMBOA was dissolved in acetone and the solution was applied to the surface of a leaf. Test leaves were treated with 500 µg DIMBOA/g fresh weight while controls received only acetone. This concentration was chosen because it allowed for comparisons with results from similar studies (see e.g. Ortego et al. 1998; Yan et al. 1999) and because it reflects naturally occurring concentrations of DIMBOA in the aerial parts of 10 d old maize seedlings (Cambier et al. 2000). After application the solvent was allowed to evaporate completely. A treated and a control leaf were placed in a Petri dish (9 cm diam.) containing a moist filter paper. A 2nd instar caterpillar of either *S. exigua* or *S. frugiperda* was placed in the centre of the Petri dish and was allowed to feed for 48 h. Petri dishes were sealed with parafilm™, placed in a transparent plastic box and kept in a climate chamber at the conditions described above. All leaves were scanned and the consumed area was calculated using the software programs Photoshop (Adobe) and Surface (C. Thiemann). Preliminary tests had confirmed a very good correlation between area and weight in barley leaves thus demonstrating that image analysis is an accurate tool for measuring the amount of barley leaf consumed. Twenty replicates were carried out.

Short term performance test

In a no-choice bioassay survival rate and biomass gain was compared for both *Spodoptera* species. Individual 2nd instar caterpillars were weighed (Sartorius MC5 microbalance) and placed in a Petri dish (5.5 cm diam.). The larvae were allowed to feed on a cube of artificial diet (300 mg) for 48 h, containing either 500 µg DIMBOA/g fresh weight or no DIMBOA. The test food was prepared by thoroughly mixing an aqueous solution of DIMBOA (200 µl) with the freshly made artificial diet (ca. 40°C). An equal amount of water was added to the control food. The Petri dishes were placed in a transparent plastic box and kept in a

climate chamber as described above. Thirty caterpillars were used per species and treatment.

Long term performance test

A second performance experiment was carried out to assess long term effects of DIMBOA on both *Spodoptera* species. Neonate larvae were kept individually in Petri dishes on DIMBOA-supplemented or DIMBOA-free diet as described above. Portions of diet were offered *ad libitum* and increased as larvae grew larger. Every second day the food was replaced by a new portion into which DIMBOA or water only was incorporated. Mixing DIMBOA into the diet was performed each time before the food was allocated to the insects to minimize the breakdown of the allelochemical. Durations of larval development, pupal weights at the first day of pupation and survival rates were recorded. Thirty to thirty-five replicates per treatment and species were carried out.

Antifungal effect of DIMBOA

The effect of DIMBOA on *S. turcica* was tested at concentrations of 0, 250 and 750 µg DIMBOA/ml agar. DIMBOA was added to V8-agar medium (40°C), poured into Petri dishes (5.5 cm diam.) and left to solidify. Four plugs were punched out from the agar of each Petri dish using a sterile cork borer (4 mm diam.). The resulting holes were equidistantly distributed. Plugs of the same size were punched out from 10 d old Petri dish cultures of *S. turcica* and placed into the holes of the DIMBOA-containing agar. Petri dishes were sealed with parafilm™ to avoid desiccation and the fungus was allowed to grow for 4 d at 20 ± 1°C. Fungal growth was calculated by tracing the outline of each mycelium onto a transparent foil. Each outline was scanned and the area was measured using the software programs Photoshop (Adobe) and Surface (C. Thiemann). The experiment was replicated four times.

Statistics

All statistical analyses were performed with the 'Statistica 7.0' software package. Feeding preferences were evaluated by Wilcoxon matched pairs test. To avoid using the traditional relative growth rate (RGR), which is associated with ratio-based statistical problems (Packard and Boardman 1999), covariate analyses (ANCOVA) were performed to measure differences in biomass gain with 'treatment' as main factor and 'initial weight' as covariable. Survival rates were evaluated by using the Chi square test while developmental time and pupal weights of larvae were compared with Student's *t*-test. Effects of DIMBOA on *S. turcica* were evaluated by two-way analyses of variance (2-way ANOVA) with 'concentration' and 'Petri dish' as variables.

Results

Feeding preference bioassay

In a dual-choice setup, caterpillars of *S. frugiperda* significantly preferred DIMBOA-treated barley leaves compared to control leaves that had been treated with acetone only (Fig. 1a; Wilcoxon matched pairs test: $P = 0.007$). The opposite was observed with larvae of *S. exigua*: caterpillars consumed more leaf tissue from barley that was devoid of DIMBOA on the surface in comparison to DIMBOA treated leaves (Fig.1b; Wilcoxon matched pairs test: $P = 0.033$).

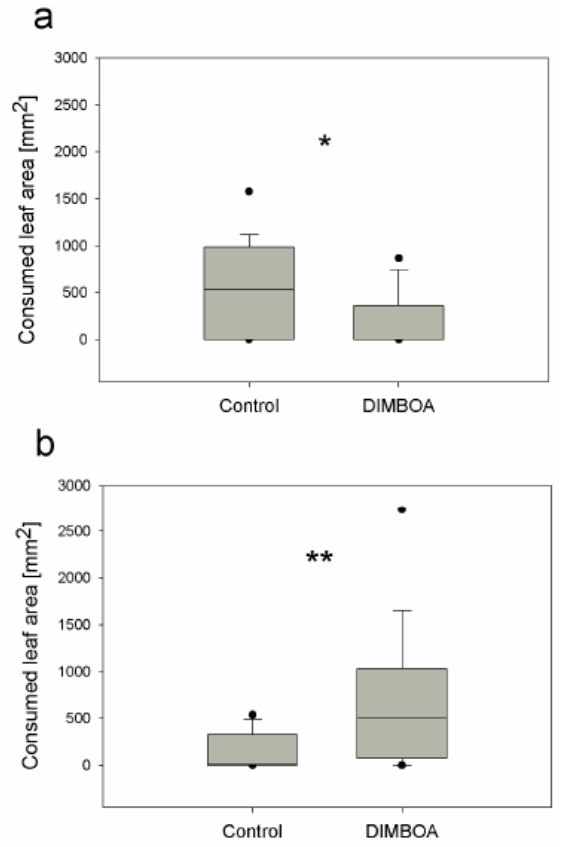

Figure 1 Feeding preferences of **a.** *Spodoptera exigua* and **b.** *Spodoptera frugiperda* on barley leaves treated with 500 µg/g DIMBOA. Boxes show median, 25th and 75th percentiles of consumed leaf areas. Whiskers indicate errors; filled circles represent minimum and maximum values. $*P < 0.05$, $**P < 0.01$. Wilcoxon matched pairs test. N = 20.

Short term performance test

The survival rate of *S. frugiperda* caterpillars was not affected by feeding on DIMBOA-containing diet (Chi square test: df = 1, $\chi^2 = 2.07$, $P = 0.150$) (Tab. 1). All larvae offered control diet survived the 48 h period, while the survival rate of *S. frugiperda* feeding on DIMBOA-containing diet cubes was 93.3%. On the other hand, caterpillars of *S. exigua* were detrimentally affected by the added allelochemical which resulted in a significantly lower survival rate compared to the control group (control vs. DIMBOA = 96.7% vs. 76.7%; Chi square test: df = 1, $\chi^2 = 5.19$; $P = 0.023$).

Analyses of covariance also revealed significant differences in biomass gain in *S. exigua*. Larvae of this species showed lower fresh weights after feeding on diet supplemented with DIMBOA in comparison to control insects (ANCOVA: initial weight, $F_{1,50} = 7.704$, $P < 0.008$; treatment, $F_{1,50} = 5.618$, $P = 0.022$). In contrast, larvae of *S. frugiperda* were not influenced by either type of diet (ANCOVA: initial weight, $F_{1,57} = 39.439$, $P < 0.001$; treatment, $F_{1,57} = 0.157$, $P = 0,693$).

Table 1 Short term performance test of noctuids reared on artificial diet containing 500 µg/g DIMBOA.

		Control	DIMBOA	*P*-level
S. exigua				
	RGR	1.48 ± 0.12	1.06 ± 0.15	*
	Survival rate	97%	77%	*
S. frugiperda				
	RGR	1.20 ± 0.07	1.29 ± 0.10	n.s.
	Survival rate	100%	93%	n.s.

Means and standard errors of relative growth rates (RGR) are given to allow for comparisons. Note that RGR were not used for testing significant differences (see materials and methods). $*P < 0.05$, n.s. = not significant. ANCOVA. N = 30

Long term performance test

As in the previous bioassay no differences in the survival rates of *S. frugiperda* feeding on either DIMBOA-supplemented or control diet were found (Chi square test: df = 1, $\chi^2 = 0.24$, $P = 0.621$) (Tab. 2). In contrast, there was also no difference in the survival rate of *S. exigua* larvae in this long term experiment (Chi square test: df = 1, $\chi^2 = 1.09$, $P = 0.297$). Overall mortality was rather high (between 34% and 50%) and rose particularly during pupation. Likewise, no differences were found in the biomasses of the pupae within both noctuid species (*S. frugiperda*: Student's *t*-test: $t = -0.828$, $P = 0.413$; *S. exigua*: Student's *t*-test: $t = -1.616$, $P = 0.116$). Caterpillars of *S. frugiperda* took the same length of time to reach the pupal stage, irrespective of the ingested diet (Student's *t*-test: $t = -0.483$, $P = 0.631$). This was in contrast to *S. exigua*, where DIMBOA significantly slowed down the development of the larvae (Student's *t*-test: $t = 2.531$, $P = 0.016$).

Table 2 Long term performance test of noctuids reared on artificial diet containing 500 µg/g DIMBOA.

		Control	DIMBOA	*P*-level
S. exigua				
	Pupal weight [mg]	126 ± 3	135 ± 4	n.s.
	Developmental time [d]	11.2 ± 0.1	11.6 ± 0.1	*
	Survival rate	50%	63%	n.s.
S. frugiperda				
	Pupal weight [mg]	216 ± 7	208 ± 6	n.s.
	Developmental time [d]	12.7 ± 0.2	12.6 ± 0.1	n.s.
	Survival rate	60%	66%	n.s.

Means and standard errors of pupal weights and developmental times are given. *$P < 0.05$, n.s. = not significant. ANCOVA and Chi-square test. N = 30-35.

Antifungal effect of DIMBOA

DIMBOA clearly inhibited the mycelial growth of the plant pathogen *S. turcica* (Fig. 2). Only the factor 'concentration' had a significant effect on mycelium size while the factor 'Petri dish' remained without influence (2-way ANOVA: concentration, $F_{2,36} = 55.863$, $P < 0.001$; Petri dish, $F_{3,36} = 0.377$, $P = 0.770$; concentration x Petri dish, $F_{6,36} = 0.915$, $P = 0.496$). A 37% reduction in fungal size was observed in cultures cultivated at 250 µg DIMBOA/ml compared to the control group (LSD: $P < 0.001$). At 750 µg DIMBOA/ml the reduction in fungal size was 58% compared to fungi grown on agar without DIMBOA (LSD: $P < 0.001$).

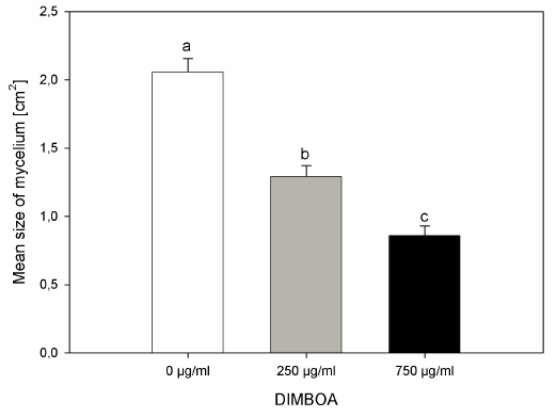

Figure 2 Growth inhibition of *Setosphaeria turcica* by DIMBOA. Means and standard errors are given. Different letters indicate statistically significant differences between treatments. 2-way ANOVA. N = 16.

Significant differences in mycelium size were also observed between fungi grown on 250 µg and 750 µg DIMBOA/ml (LSD: $P = 0.001$).

Discussion

This study showed that DIMBOA had a differential impact on two closely related generalist herbivore species. Caterpillars of *S. frugiperda* preferred barley leaves when supplemented with DIMBOA. This suggests that *S. frugiperda*, with its affinity to maize (Sparks 1979), could use DIMBOA for host recognition and as a feeding stimulant. In contrast, *S. exigua*, which is often found on dicotyledonous plants such as cotton (*Gossypium hirsutum*), pigweed (*Amaranthus spp.*), or celery (*Apium graveolens*) (Eveleens et al. 1973; Berdegue et al. 1998; Showler 2001), was deterred by DIMBOA-treated leaves. The findings also correlate with the effects DIMBOA had on biomass gain and mortality in the short term performance test. Larvae of *S. frugiperda* coped well with the allelochemical in the diet. They did not differ in weight from those feeding on DIMBOA-free diet and they had equal survival rates. In *S. exigua,* DIMBOA was found to be detrimental: larvae gained less weight and had a significantly higher mortality rate compared to controls. Although the outcome of the long term performance test was not congruent in all aspects to the short term assay, it confirmed in principle the overall picture that *S. frugiperda* appears to be better adapted to the toxic effects of DIMBOA than *S. exigua*. The survival rates and the pupal biomasses of *S. exigua* feeding on DIMBOA-diet and control diet did not differ in the long term assay. It is conceivable that the high overall mortality of the larvae in this experiment superposed any diet effects. Nevertheless, DIMBOA had a significant detrimental effect on the duration of development of *S. exigua* caterpillars. Plant secondary metabolites have often been found to exert such sub-lethal effects on

herbivorous insects. The consequence may be that the herbivore remains in the vulnerable larval stage for a prolonged time, so overall mortality might be high due to predators, parasitoids and pathogens (Benrey and Denno 1997; Rostás and Hilker 2003; Cornelissen and Stiling 2006). Testing this so-called slow growth-high mortality hypothesis (Williams 1999) with *S. exigua* could be rewarding.

Comparable studies with other lepidopteran pests of maize showed that in general DIMBOA acts as an effective allomone. In the best studied species, *Ostrinia nubilalis,* diet containing 500 µg/g DIMBOA significantly reduced larval growth and survival rate (Campos et al. 1989). Similar findings were described for another stalk borer, *Sesamia nonagrioides* (Ortego et al. 1998). In this herbivore, ingestion of DIMBOA had no short term effects but rearing caterpillars for two larval stages on DIMBOA-diet significantly reduced the relative growth rate. Adverse effects on development and a preference of DIMBOA-free leaves were also reported for the Asian corn borer, *O. furnacalis* (Yan et al. 1999).

The detrimental effects of DIMBOA on herbivores in general and on *S. exigua* in our study may result from its antifeedant properties as well as from toxic effects. DIMBOA is known as an inhibitor of digestive proteases such as trypsin and chymotrypsin and of detoxification enzymes (Ortego et al. 1998) but it may also inactivate mitochondrial ATP synthesis (Niemeyer et al. 1986). Further experiments on the physiological level are needed to elucidate the differences in toxicity between both noctuid species.

The fungus bioassay confirmed earlier studies which correlated DIMBOA content in maize inbred lines with resistance against *S. turcica* and which showed that it can affect spore germination (Couture et al. 1971; Long et al. 1975). Both concentrations (250 µg and 750 µg DIMBOA/g agar) inhibited the growth of fungal hyphae in a dose-dependent manner.

Concluding from these results high DIMBOA content in maize should contribute well in the control of *S. turcica* but only moderately or negligibly in the control of *S. exigua* and *S. frugiperda*, respectively.

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