Distribution and Variations of Three 1,4-Benzoxazin-3-ones in Maize Induced by the Asian Corn Borer, *Ostrinia furnacalis* (Guenée)

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Contents of three 1,4-benzoxazin-3-ones in tissue samples from different parts (young leaf, second leaf, old leaf, stem and root) of young maize plants of 4-leaves stage, fed by the third instar larvae of the Asian corn borer, *Ostrinia furnacalis* (Guenée), were analyzed by high-performance liquid chromatography-mass spectroscopy (HPLC-MS). Samples were taken immediately (set A) or 48 h (set B) after larvae had fed on the second leaf for 48 h. The three 1,4-benzoxazin-3-ones investigated in our experiments were 2,4-dihydroxy-7-methoxy-1,4(2*H*)-benzoxazin-3-one (DIMBOA), 2,4-dihydroxy-1,4(2*H*)-benzoxazin-3-one (DIBOA) and 2-hydroxy-7-methoxy-1,4(2*H*)-benzoxazin-3-one (HMBOA). In samples of set A, the levels of DIMBOA and HMBOA were significantly lifted in the old leaf (L3) and young leaf (L1), respectively, while amounts of these two chemicals in other plant parts were not significantly different between larvae-fed plants and intact plants. Concentrations of DIBOA in each plant part remained unchanged. In samples of set B, no concentration differences for any of these three 1,4-benzoxazin-3-ones between larvae-fed plants and controls were observed in any plant part. The feeding of the Asian corn borer seems to have limited effects on induction of these three 1,4-benzoxazin-3-ones in young maize plants of the variety investigated.

Key words: 1,4-Benzoxazin-3-ones, *Ostrinia furnacalis*, Maize

Introduction

The Asian corn borer (ACB), *Ostrinia furnacalis* (Guenée) (Lepidoptera: Pyralidae), is a serious pest in China's maize production (Wen *et al.*, 1992). Among the tools used for control of ACB, plant resistant variety breeding has played important roles. A series of benzoxazinones (Bxs), found in many species of Gramineae (Hofman and Hofmanová, 1969; Niemeyer, 1988) as well as in some species of dicotyledonous plants (Pratt *et al.*, 1995), are important secondary metabolites of plants in resistance against insects, fungi and algae as well as in herbicide tolerance and allelopathic purposes (for review, see Niemeyer, 1988). These chemicals are present in intact plants as 2-β-*O*-D-glucosides; and after tissues are injured, the glucosides are hydrolized by β-glucosidase to release the corresponding aglycones that are toxic to insects (Massardo *et al.*, 1994). The presence and contents of Bxs are variable with plant species. 2,4-Dihydroxy-7-methoxy-1,4(2*H*)-benzoxazin-3-one (DIMBOA) is a main component in maize, wheat and *Coix* spp., but a minor in rye; 2,4-dihydroxy-1,4(2*H*)-benzoxazin-3-one (DIBOA) is the main constituent in rye with low concentrations in maize; 2-hydroxy-7-methoxy-1,4(2*H*)-benzoxazin-3-one (HMBOA) was found in high concentrations in maize and *Coix* spp. but in minute amounts in wheat and rye (Hofman and Hofmanová, 1969). The structures of these three chemicals are shown in Fig. 1. There are also other minor Bxs in the grasses (Woodward *et al.*, 1979; Niemeyer, 1988; Hashimoto and Shudo, 1996). The biosynthesis pathway of Bxs has been studied and some genes of catalytic enzymes have been located in wheat and maize (Niemeyer and Jerez, 1997; Frey *et al.*, 1997, 2003).

DIMBOA is a broad-spectrum chemical in plants against aphids (Givocich and Niemeyer, 1996; Escobar *et al.*, 1999), corn borers (Klun *et al.*, 1970; Yan *et al.*, 1995, 1999; Ortego *et al.*, 1998), ect. DIBOA was repored to play some role against the Russian wheat aphid (*Diuraphis noxia*) and the greenbug (*Schizaphis graminum*) in wild Poaceae (Gianoli and Niemeyer, 1998). Few literature was found on the biological properties of HMBOA and other minor Bxs, so their biological functions are still not well understood.

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Although there were some researches on toxic and antifeedant effects of DIMBOA on ACB (Yan *et al.*, 1995, 1999), no work has been done on the induction of Bxs in maize by ACB. In the present research, concentrations of DIMBOA, DIBOA and HMBOA in the leaves, stems and roots of maize plants were quantified after feeding by ACB larvae, in the attempt to understand distribution patterns and changes of these chemicals in the maize plants under induction of ACB.

Materials and Methods

Plants and insects

Seeds of maize (*Zea mays* L.) variety *Nongda-108* were provided by China Agricultural University, Beijing. Seeds were sowed in mimic soil in plastic pots (12 cm in diameter and 10 cm in height) and then kept in a growth chamber (HPG-280B, Harbin Donglian Electronic Technique Co., Ltd.) under (28 ± 1) °C and 300 Lux with a 16 h:8 h (L:D) photoperiod and ca. 75% relative humidity. After the seedlings emerged, only one single plant was kept per pot. Plants at four-leaves stage (3 fully expanded leaves and the cotyledon) with about 35 ~ 45 cm in height were used for experiments. The cotyledon was not used for samples.

Egg masses of ACB were obtained from Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing. Larvae were reared on a semi-artificial diet (Zhou *et al.*, 1980) under (28 ± 1) °C with a 16 h:8 h (L:D) photoperiod and 75% relative humidity in a growth chamber. The 3rd instar larvae were used for all experiments.

Infection experiments

Plants were chosen randomly as treatments and controls, respectively. In the treatment group, two 3rd instar larvae, having starved for 2 h, were confined within a clip plastic cage (2.5 cm in diameter, 2.2 cm in height) on the upper part of the second fully extended leaf (L2) of each plant for 48 h; in the control group there were no larvae in the cage. The plants with/without caged larvae were kept in the growth chamber under the same conditions as mentioned above. Plants and insects in experiments were checked daily, and when the leaf surface inside the cage was almost consumed, the cage was moved a little (1 or 2 cm) towards the middle of the leaf to provide more food to the larvae. After samples were taken (see below), the leaf that the larvae fed on was removed and the amount of consumed area was measured. The average area consumed was about 30 ~ 40% of total leaf surface. The experiments were repeated for 16 times with each sample measured for 3 times.

Sample preparation

There were two sets of samples in the experiments. Samples of set A were taken immediately after larvae were removed from the plants; and samples of set B were obtained 48 h after larvae were removed. Tissue samples [20–50 mg fresh weight (fr. w.)] from different plant parts, *i.e.*, the first (young) leaf (L1) (from top of the plant), the second leaf (L2), the third (old) leaf (L3), stem (S), and root (R), were taken with sterilized scissors and homogenized with 320 μl dd-H_2O using a mortar and pestle. The homogenized samples were washed with 2 × 320 μl dd-H_2O. The aqueous extract was left at room temperature for 15 min allowing the release of Bxs from their glucosides (Nicol and Wratten, 1997). Then the pH value of the solution was adjusted to 3 with 40 μl 0.1 N H_3PO_4. The extract was then centrifuged at 12,000 rpm for 15 min. The supernatants were stored at – 20 °C in a refrigerator until used.

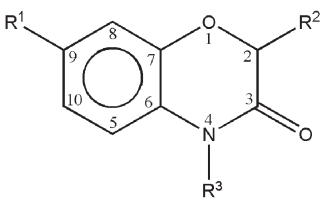
Chemicals analysis

DIMBOA, DIBOA and HMBOA were separated and identified by high-performance liquid chromatography-mass spectroscopy (HPLC-MS). 20 μl of the sample were directly injected into a HPLC-MS instrument (Aglient 1100 series HPLC equipped with a diode array detector and mass spectrometer) with a Sepax HP-C18 column (250 × 4.6 mm). A constant solvent flow of 1 ml/min was used with solvents A (MeOH) and B (0.02% TFA in 1 l water) at the ratio of 35% ~ 40% A from 0 to 6 min, 40% A from 6.1 to 9 min, 95% A from 9.1 to 11 min and 35% A from 11.1 to 18 min. Identification of 1,4-benzoxazin-3-ones was made according to UV absorption (from 200 nm to 400 nm) and mass characteristics in comparison with authentic chemicals. The quantification of the chemicals was carried out through UV absorption peak areas at 263 nm. Each sample was injected three times to get a mean value.

Results

Abundance of three 1,4-benzoxazin-3-ones in intact maize plants

The distribution of three 1,4-benzoxazin-3-ones (Fig. 1) varied in different plant parts (Fig. 2). The results showed that in the aerial parts of uninjured maize plants, higher DIMBOA levels were found in young leaves (L1), while higher concentrations of DIBOA or HMBOA were found in older leaves (L3 or L2). The highest contents of DIMBOA and DIBOA in the plants were found in roots. Among the three Bxs studied, DIMBOA was the most abundant in each plant part except L3 where HMBOA was higher in content, whilst DIBOA was only minor in all parts.



	R^1	R^2	R^3
DIMBOA:	OMe	OH	OH
DIMBOA-Glc:	OMe	O-Glc	OH
DIBOA:	H	OH	OH
DIBOA-Glc:	H	O-Glc	OH
HMBOA:	OMe	OH	H
HMBOA-Glc:	OMe	O-Glc	H

Fig. 1. The structure of 1,4-benzoxazin-3-ones.

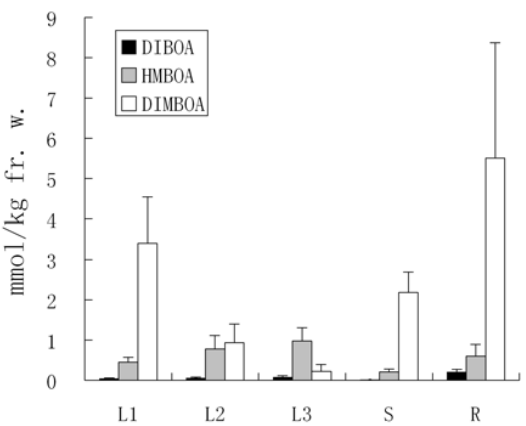


Fig. 2. Contents of DIBOA, HMBOA and DIMBOA in different parts of intact maize plants (four-leaves stage). Samples were taken from the first/young leaf (L1), the second leaf (L2), the third/old leaf (L3), the stem (S) and the root (R).

1,4-Benzoxazin-3-ones content changes induced by feeding of ACB

After 48 h feeding by the third instar larvae of ACB on L2 (set A), HMBOA increased significantly in L1 ($P = 0.047$), but no remarkable increments were observed in other plant parts. The DIMBOA amount increased very significantly in L3 ($P = 0.014$), and the increments in other parts were inconspicuous (Fig. 3). No significant differences in DIBOA amounts were observed in all plant parts between the test and control group.

The contents of the three Bxs in samples (set B) taken 96 h after initial feeding of ACB (48 h after removing of the larvae from the plants) dropped to very low levels, but the distribution patterns were almost the same as in samples of set A. There were no significant differences found between the test and control group for the three Bxs studied (Fig. 4).

Discussion

Distribution pattern of three 1,4-benzoxazin-3-ones

In coevolution of the plant-insect interations, plants have developed chemical defense strategies including storage of precusors of defense chemicals in the tissues where most probably attacks of pests may occur. For ACB, younger larvae feed on tender leaves and then they bore into stems. Therefore, it is reasonable to find higher DIMBOA contents in young leaves (L1) and stems (S) in the aerial parts of intact maize plants in our experiments (Fig. 2). However, our results of DIMBOA in roots are different from what was reported in the literature. It was reported that the DIMBOA content was always higher in aerial parts than in roots (Cambier *et al.*, 2000), but in our experiments, very high content of DIMBOA was found in roots compared to other parts investigated in the uninjured plant (Fig. 2). High content of DIMBOA accumulated in roots may be used by plants for allelopathic purposes (Friebe *et al.*, 1995; Wu *et al.*, 2002), metal nutrition uptake (Pethő, 2002), and also possibly for defending soil pests.

The distribution pattern of HMBOA was quite different from that of DIMBOA in uninjured maize plants. It accumulated more in older leaves than in the young leaves (L1) (Fig. 2). In L3, the

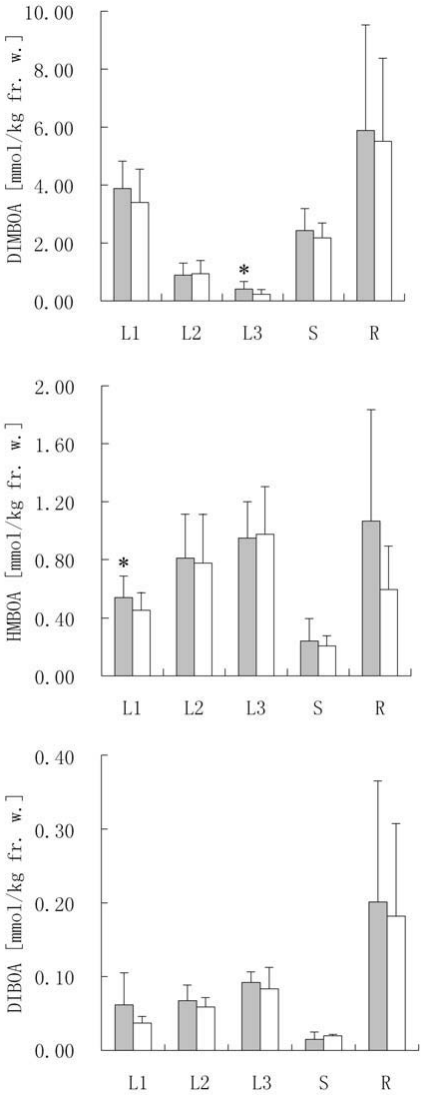


Fig. 3. Contents of DIBOA, HMBOA and DIMBOA after induction by 48 h feeding of the Asian corn borer. The shaded bar refers to the test group, and the empty bar refers to the control group. Samples were taken from the first/young leaf (L1), the second leaf (L2), the third/old leaf (L3), the stem (S) and the root (R). The samples were taken immediately after removal of the larvae. Asterisk (*) mean significant difference between test and control groups at a level of 0.05.

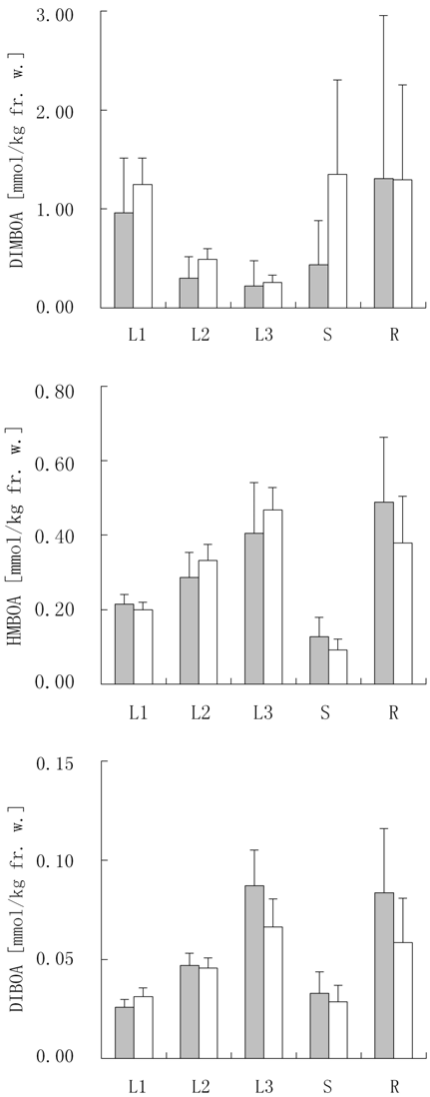


Fig. 4. Contents of DIBOA, HMBOA and DIMBOA in maize plant 48 h after removal of the ACB larvae. The shaded bar refers to the test group, and the empty bar refers to the control group. Samples were taken from the first/young leaf (L1), the second leaf (L2), the third/old leaf (L3), the stem (S) and the root (R).

HMBOA content was even higher than the DIMBOA content. HMBOA was detectable after about six days after maize germination, much later than DIMBOA and DIBOA (Cambier *et al.*, 2000), so it might be converted from other Bxs while tissues get aging in maize. DIBOA was similar to HMBOA in distribution pattern, but in very low contents.

Induction of three 1,4-benzoxazin-3-ones by ACB

The Bxs, especially DIMBOA, has been extensively researched on content changes induced by different insects or artificial leaf damage in maize. Feeding of *Sesamia nonagrioides* for 60 h in-

creased the DIMBOA contents of 42% and 96% in the leaves of two maize inbred lines (Gutierrez *et al.*, 1988); artificial leaf damage increased DIMBOA concentration significantly (Morse *et al.*, 1991). Aphids also induced the DIMBOA accumulation in wheat (*e.g.*, Niemeyer *et al.*, 1989; Gianoli and Niemeyer, 1997b). In our results, ACB feeding for 48 h could also induce an increase of DIMBOA and HMBOA contents (Fig. 3). Several reasons for DIMBOA and HMBOA induction by ACB feeding could be assumed. Firstly, the increment of these two chemicals could come from the transformation of other Bx-Glcs locally. Niemeyer (1988) proposed that HMBOA could be converted to DIMBOA by hydroxylation. If this is the case, the content of HMBOA should decrease while the DIMBOA content increases. But HMBOA or DIBOA were not found to become lower in contents (Fig. 3). Was DIMBOA transformed from other Bxs other than HMBOA or DIBOA? Secondly, these two chemicals were transported from other parts of plants. It was reported that the induced Bxs might rely on the transportation of defense chemicals from other parts of the plant to the aphid-infested site in wild wheat (Gianoli and Niemeyer, 1997a). However, no remarkable decreases of DIMBOA or DIBOA in other plant parts were observed in our present study. Thirdly, these two chemicals were synthesized *de novo*. Further work is needed to investigate induction mechanisms of these chemicals.

Even though relatively high contents of HMBOA were found in intact and injured maize plants in our experiments (Fig. 2), there has been little information about its biological properties. It is still unclear if HMBOA is just a byproduct in the DIMBOA synthesis pathway without any biological property or plays some independent biological roles in plant defense. It is very necessary to investigate more of its biological properties in the further research.

Results of timing course experiments were sometimes variable (Korth and Dixon, 1997). In our experiments, contents of these three Bxs were also variable, probably resulting from plant and insect individuals, or from difference in consumed areas of leaves, *etc.* In addition, contents of the three Bxs in samples of set B that were taken 48 h after larvae were removed from the plants in both test group and control group reduced rapidly compared to those of set A. The drop of Bxs with maize plant development was believed to be the normal trend (Gutierrez *et al.*, 1988; Cambier *et al.*, 2000). Morever, in samples of set B, no significant differences were found between the test and control groups. It seemed that when the stimulation from insect feeding was off, Bx levels returned gradually to normal. Such a trend may be the strategy of plants to save energy when other defense mechanisms (especially physical defense) have developed with the plant growth.

In general, Bxs chemical responses in maize plants to ACB feeding were systematic because induced DIMBOA or HMBOA was found in the parts other than the feeding site. DIMBOA and HMBOA contents only increased significantly at some parts of the plant after feeding by ACB, implying that the induction of Bxs in our maize variety by feeding of ACB was not remarkable. Plant inbred lines can influence induction greatly and insects' salivary components play important roles in plant chemical inductions (Musser *et al.*, 2002). Analysis of components in saliva or regurgitants of ACB larvae might elicit the role of ACB in chemical induction of maize.

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