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1,4-BENZOXAZINE DERIVATIVES IN PLANTS: ABSENCE OF 2,4-DIHYDROXY-7-METHOXY-2H-1,4-BENZOXAZIN-3/4H/-ONE FROM UNINJURED *ZEA MAYS* PLANTS

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Abstract—Free 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3/4H/-one (DIMBOA) could not be detected in uninjured corn plants. The resistance of plants is thus not determined by the presence of free aglucone in the uninjured plant, but corresponds to a high content of glucosides of 1,4-benzoxazine in the resistant lines and by the capacity to release from them antifungally active aglucones.

INTRODUCTION

THE ANTIFUNGAL substance 2(3)-benzoxazolinone (BOA) was isolated from rye,[1] and 6-methoxy-2(3)-benzoxazolinone (MBOA) from *Coix lachryma* Jobi,[2] corn[3,4] and wheat.[3] It was found later that these substances are not natural but originate through the degradation of 1,4-benzoxazines which appear in bound form as glucosides in plants.[5–7] The appearance of aglucones in an injured plant is effected by the presence of the hydrolytic enzyme β-glucosidase.

In earlier experiments, no attention was paid to the problem of whether free aglucone occurs in uninjured plants, especially in resistant lines. This problem was dealt with by Wahlroos and Virtanen in connection with the occurrence of MBOA in corn;[8] they conclude that "MBOA is not present in fresh plant tissue even of the resistant maize inbred W 22, but that the free aglucone is present in appreciable concentration in this maize variety and, accordingly, may vary widely in different maize strains. The aglucone which has antifungal properties may thus be the resistance factor in maize." From this, it may be presumed that free DIMBOA aglucone is present in the plant. Having considered the time sequence of the publications,[8,9] the absence of aglucone in the Early Albert line[9] and its presence in the naturally resistant line W 22,[8] we presumed the resistance of corn lines to correspond to the amount of free DIMBOA in the plant.

The presence of DIMBOA being considered as proved, its increased concentration in highly resistant corn lines was assumed. However, our experiments led to different conclusions. We isolated new 1,4 benzoxazine glucosides, viz. 2-(2-hydroxy-2H-1,4-benzoxazin-3/4H/-one)-β-D-glucopyranoside,[10] 2-(2,7-dihydroxy-2H-1,4-benzoxazin-3/4H/-one)-β-D-

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[8] Ö. WAHLROOS and A. I. VIRTANEN, *J. Pharm. Sci.* **53**, 844 (1964).
[9] A. I. VIRTANEN and Ö. WAHLROOS, *J. Pharm. Sci.* **52**, 713 (1963).
[10] J. HOFMAN and O. HOFMANOVÁ, *European J. Biochem.* **8**, 109 (1969).

glucopyranoside[11] and 2-(2-hydroxy-4,7-dimethoxy-2H-1,4-benzoxazin-3-one)-β-D-glucopyranoside;[12] the isolation of 2-(2,4-dihydroxy-7,8-dimethoxy-2H-1,4-benzoxazin-3/4H/-one)-β-D-glucopyranoside* will be published later. Aglucones originating through an injury of the plant formed a chromatographically uniform fraction, which was considered as one substance[7]—DIMBOA, possibly polluted by MBOA.

We analysed 11 corn lines (WF 9, B 14, B 37, B 52, R 101, W 22, Hy, OH 43, B 49, OH 45, CI31A) with varying resistance against the European corn borer[13] for their content of 1,4-benzoxazine glucosides and free aglucones. We found that the content of aglucones in extracts is very low and that there are no differences among the lines. In spite of the content of glucosides in individual plants of the same line being almost the same (low variability inside the line), the amount of aglucones fluctuated. This finding suggested that most of the plants must have been injured, at least partially, during the manipulation and that the amount of aglucones therefore seemed to be a measure of that injury. Our present experiments were aimed at confirming this conclusion.

While searching for aglucones in uninjured plants, we started from the facts that a corn plant contains relatively stable glucosides and also a very active enzyme β-glucosidase. It was necessary to choose a method that would record the conditions in the intact plant and that would avoid enzymic hydrolysis.

These conditions were fulfilled in our method of stabilization and extraction of 1,4-benzoxazines as their glucosides.[10] Homogenization and extraction is complete and the extract is not subject to enzymic attack. If aglucones are present, they are not further broken down during extraction and isolation.

RESULTS

We examined the highly resistant line CI31A,[13] the naturally resistant line W 22[8] and the line 'Bílý koňský zub' using three different experimental procedures.

In the first experiment, most of the sand was removed from the roots of corn seedlings and plants were fixed in liquid nitrogen (see Experimental). After extraction and separation of the glucosides from aglucones by means of partition chromatography, their amounts were determined. The aglucone content was one-tenth that in the analysis of one individual plant which was much more injured during the removal of sand from the roots (Table 1).

In the second experiment, unwanted injury was avoided by removing the sand in a slow flow of water. Seedlings with roots enfolded by sand were frozen again by liquid nitrogen and homogenized. In this case, too, free aglucones were found in both lines W 22 and CI31A, but in a smaller amount than in the first experiment in the line with low resistance. These amounts were so small that it was impossible to denote the plant as being resistant or sensitive according to the amount present.

In the third experiment, manipulation of the plants was eliminated by cultivating them directly in the mortar where later all the contents were frozen. Under these conditions, no free aglucones were found.

Owing to the relatively lower activity of β-glucosidase and considerable supporting network protecting the plant from being injured, it was easier to prove the absence of aglucones in large plants than in seedlings.

* NMR: d_6 — DMSO, ref. TMS, $\delta_{c-7} = 3{\cdot}80$, $\delta_{c-8} = 3{\cdot}83$; m.p. 149–151°.

[11] J. HOFMAN, O. HOFMANOVÁ and V. HANUŠ, *Tetrahedron Letters* 5001 (1969).
[12] J. HOFMAN, O. HOFMANOVÁ and V. HANUŠ, *Tetrahedron Letters* 3213 (1970).
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TABLE 1. 1,4-BENZOXAZINE CONTENTS IN CORN PLANTS EXTRACTED UNDER DIFFERENT CONDITIONS

Procedure	Corn line	No. plants	Glucosides	Aglucones	Ratio glucosides aglucones
			μ moles/g fr. wt.		
	'Bílý koňský zub'	1	4·5	0·11	41
(I)	'Bílý koňský zub'	30	4·0	0·0105	380
(II)	W 22	33	6·3	0·0128	490
	CI31A	35	6·8	0·0083	820
(III)	CI31A	25	*ca.* 6·5	—	—
External leaves of mature corn plant (CI31A)		—	1·8	—	—

In order to complete the study, the influence of aglucones upon the growth of seedlings was examined. Plants were grown in the medium containing a smaller amount of aglucone than that present in the plant in the form of glucosides. Since the plants stopped growing and died, it follows that free aglucones are toxic and do not occur naturally.

DISCUSSION

On the basis of the experiments described above, it is possible to conclude that free DIMBOA is absent from uninjured plants. Other possible algucones of the known glucosides present[10–12] are also presumably absent. From the effect of these aglucones on the growth of corn seedlings, it is evident that it would not be suitable for the plant to contain a large amount of aglucones to control resistance. Thus, the resistance of plants is not determined by the presence of free aglucones but corresponds to a high content of 1,4-benzoxazine glucosides present in resistant plants and to their ability to split quickly into antifungally effective aglucones.

EXPERIMENTAL

Seedlings of the naturally resistant corn line W 22, the highly resistant line CI31A and the line 'Bílý koňský zub' were cultivated in a greenhouse on the sand at 20–23° for 3 weeks in 20 cm dishes (with 30–35 plants/dish). Analyses were carried out with all plants from one dish at a time as soon as they reached the height of 25 cm. Mature corn plants of the line CI31A were cultivated in the field from June to August and reached the height of 120–130 cm.

Procedure I

30 corn seedlings of the line 'Bílý koňský zub' were taken and the greater part of the sand was removed from the intertwined roots by a flow of water and the seedlings were put in excess liquid N_2 in a big mortar, crushed and pulverized.

No liquid N_2 may be added during pulverization since the homogenate would be dispersed and the glucosides split enzymatically due to higher temperature.

The homogenate was suspended in two litres of acetone and the acetone extract was filtered and evaporated to a small vol. at 15°. By preparative descending partition chromatography on thick paper (*n*-BuOH–EtOH–H_2O, 5:1:4), the glucosides of 1,4-benzoxazine (R_f 0·2–0·4) were separated from aglucones (R_f 0·8); detection was by means of their absorbance in u.v. light of 253·7 nm. The glucosides were eluted with water and the aglucones with 70% EtOH.[14] A sample of the glucoside fraction was separated by gel filtration on Sephadex G-10.[10] Due to the small amount of aglucones present, they were determined spectrophotometrically as a mixture and related to DIMBOA, which is their dominant component.

Procedure II

33 plants of the line W 22 and 35 plants of CI31A were experimented upon. Sand (about 80 per cent of it) was removed by a very slow flow of water. The plants with roots enfolded by sand were frozen again, crushed and analyzed by procedure I.

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Procedure III

Corn seedlings of the line CI31A were cultivated directly in the mortar on a layer of sand 2cm high. When they reached the height of about 25 cm, they were frozen in excess liquid N_2, pulverized, and the analysis carried out as in procedure I. Under these conditions, no free aglucones were found.

Mature Corn Plants

In order to prove the presence or absence of aglucones, external leaves were chosen which are most suitable with regard to the size of the plant. Weighed, cut off, 60 cm long external leaves of the CI31A line were successively dipped from two thirds into liquid N_2 and frozen parts pulverized. Extract was analyzed by the standard procedure.

The Effect of 1,4-*Benzoxazine Aglucones upon the Growth of Corn Seedlings*

3 cm-long corn seedlings were placed on the bottom of a glass dish with filter paper dipped in 5 mm layer of 0·1% solution of corn aglucones in water. Aglucones were dissolved in a few drops of ethyl alcohol and diluted with water. After 24 hr, the growth of corn seedlings was completely arrested and in 5 days the plants died.

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