

# Localization of Benzoxazinones that Occur Constitutively in Wheat Seedlings

Hajime Iwamura<sup>a</sup>, Eri Nakagawa<sup>a</sup>, and Nobuhiro Hirai<sup>b</sup>

<sup>a</sup> Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University, Kyoto 606-01, Japan

<sup>b</sup> Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto 606-01, Japan

Z. Naturforsch. **51c**, 807–812 (1996); received July 8/August 12, 1996

Benzoxazinones, Ferric Chloride Staining, Gramineae, Wheat Seedlings

Occurrence and localization of novel antimicrobial and antifeeding compounds in wheat, 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), and their glucosides, were examined by staining wheat plants (*Triticum aestivum* L.) in the juvenile stage of growth by ferric chloride. The methanol extracts of the stained plant tissues gave a characteristic blue color, which was shown by spectroscopic and chromatographic analyses to be exclusively due to benzoxazinones. When ferric chloride was applied to the root in the seedlings, the blue color immediately developed, the staining being strongest at the tip region and becoming lighter towards the basal part. The staining pattern of the radicle in the pre-emerging seed was similar to that in the root, but the coleorhiza was not stained. Little staining was observed in the epidermal layer of the leaf sheath in the shoot but the underlying tissue was stained strongly. The foliage leaf folded in the sheath was also stained, but less intense than the sheath tissue. It is suggested that the DIBOA and DIMBOA are produced within the stained region of the leaf and root. Together with previous findings that the benzoxazinones appear constitutively in wheat during the juvenile stage of growth, their localized occurrence in the tissues exposed to microbial and insect attacks suggests that they act as defense compounds during this vulnerable plant stage.

## Introduction

Benzoxazinone glucosides, 2,4-dihydroxy-1,4-benzoxazin-3-one glucoside (DIBOA-G), and its methoxy analog, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside (DIMBOA-G), are known to occur in Gramineae including maize, wheat and rye (Niemeyer, 1988). Upon infection or by an insect attack, they are rapidly hydrolyzed to produce the aglycones DIBOA and DIMBOA with antimicrobial and antifeeding activities, and have been implicated to be involved in the resistance of the plants to pathogens and insects (Niemeyer, 1988; Gutierrez *et al.*, 1988; Niemeyer *et al.*, 1989). In some cases the degradation products of DIBOA and DIMBOA, 2-benzoxazolinone (BOA) and 6-methoxy-2-benzoxazolinone (MBOA), occur together with the precursors, and are also active as antibiotics and antifeedants (Niemeyer, 1988; Wahlroos and Virtanen, 1959; Baker and Smith, 1977). However, most of these studies have

been concentrated on autotrophic seedlings or adult plants.

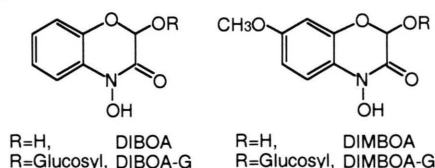


Fig. 1. Structures of benzoxazinones.

Recently, we have found that DIBOA-G and DIMBOA-G occur in both the shoot and root of germinating wheat (*Triticum aestivum* L. cv. Asakazekomugi), followed by the aglycones DIBOA and DIMBOA (Nakagawa *et al.*, 1995). These compounds, especially aglycones, attain a maximum level soon after the germination and then disappear gradually as the plant begins autotrophic growth. The timing of the appearance and disappearance is little affected by pathogen infections or by wounding of the plant with a razor blade. These results suggested that the antimicrobial and antifeeding compounds appear according to a schedule as defense compounds in the vulnerable, juvenile stage of growth. The levels of DIBOA

Reprint requests to Dr. H. Iwamura.  
Telefax: +81-75-753-6408.

0939-5075/96/1100-0807 \$ 06.00 © 1996 Verlag der Zeitschrift für Naturforschung. All rights reserved.

D



Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht: Creative Commons Namensnennung-Keine Bearbeitung 3.0 Deutschland Lizenz.

Zum 01.01.2015 ist eine Anpassung der Lizenzbedingungen (Entfall der Creative Commons Lizenzbedingung „Keine Bearbeitung“) beabsichtigt, um eine Nachnutzung auch im Rahmen zukünftiger wissenschaftlicher Nutzungsformen zu ermöglichen.

This work has been digitalized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution-NoDerivs 3.0 Germany License.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition “no derivative works”). This is to allow reuse in the area of future scientific usage.

and DIMBOA have been found sufficient to defend the plant against an attack by a pathogen or insect even if the compounds are assumed to be distributed uniformly in the tissues (Nakagawa *et al.*, 1995). Actually the aglycones may be localized mainly in those parts of the plant exposed to microbial and insect attacks, or more in the cortical than in the internal tissues.

These benzoxazinones are known to react with ferric chloride to form complexes with characteristic bluish color, and this has been utilized for their identification and quantitation in plant extracts (Hamilton, 1964; Corcuera *et al.*, 1978; Woodward *et al.*, 1979; Argandoña *et al.*, 1981). In this study, we utilized this feature to stain the plants in the pre-germinating and juvenile stages of growth and observe their presence and localization in the tissues.

## Materials and Methods

### *Preparation of benzoxazinones*

DIBOA-G, DIMBOA-G and DIBOA were isolated from wheat seedlings, and DIMBOA was prepared by  $\beta$ -glucosidase hydrolysis of DIMBOA-G, as reported previously (Nakagawa *et al.*, 1995). Briefly, shoots of 11,000 seedlings grown for 40 hr as described below were extracted with hot 2% acetic acid for 10 min. The solution was filtered and extracted with *n*-butanol, and the extract was concentrated under reduced pressure. The residue was chromatographed on a silica gel column that was eluted with chloroform containing methanol and 1% acetic acid. The fractions eluted with 3 and 10% methanol were combined and purified by HPLC using an ODS column (A-323, YMC Co.) that was eluted with 0.3% acetic acid-30% methanol-water (v/v) with monitoring at 254 nm to give 14, 13 and 9 mg of DIBOA, DIBOA-G and DIMBOA-G, respectively. To obtain DIMBOA, a mixture of DIMBOA-G (4.4 mg) and 5 mg of  $\beta$ -glucosidase (22 units, Sigma) in 0.5 ml acetic acid-sodium acetate buffer (100 mM, pH 5.1) was incubated at 37 °C for 4 hr, diluted with water, made acidic with 1 M HCl and extracted with ethyl acetate, and the extract was evaporated to dryness. The yield was 2.6 mg. The compounds were identified by  $^1\text{H}$  NMR, MS and UV spectra (Woodward *et al.*, 1978; Lyons *et al.*, 1988), and the structures are shown in Fig. 1.

### *Seedlings culture*

Seeds of wheat were placed in a Petri dish (9 cm i.d. x 1.5 cm height) containing three layers of filter paper and 20 ml of distilled water. The seeds were incubated at 25 °C for a 12-hr daily period of illumination with fluorescent lamps (30 W m<sup>-2</sup>).

### *Staining by ferric chloride*

Plant material was stained with a few drops of a solution consisting of 1.0 g FeCl<sub>3</sub>·6H<sub>2</sub>O dissolved in a mixture of 10 ml of 95% ethanol and 100  $\mu$ l of 1.5 M HCl, and the development of the color was observed under a stereomicroscope. For the extraction of the complexes to measure UV spectra, shoots from 24-hr old seedlings and roots cut out from 72-hr old seedlings (50 mg each) were stained for 2 min with the ferric chloride solution diluted fourfold by methanol to minimize the spectral disturbance by ferric chloride that gives a yellow color and has an absorbance maximum at 365 nm. The stained material was immersed in 1 ml of methanol for 3 min, the mixture was centrifuged at 4,500 x *g* for 5 min, and the supernatant was subjected to spectral measurements. The solutions of the authentic benzoxazinone complexes were prepared by dissolving 0.3 mg each of the compounds isolated as above in 3 ml methanol containing 2  $\mu$ l of the ferric chloride solution diluted fourfold by methanol. Spectra were recorded on a Hitachi 220 spectrophotometer (Tokyo, Japan). TLC of the extracts was performed on a silica gel plate (Merck, silica gel 60 F<sub>254</sub>, layer thickness 0.20 mm) with chloroform: methanol: acetic acid = 16: 4: 1 (v/v).

### *Water content of the plant tissue*

Plant material (11–14 mg) was dried at 90 °C for 1 hr in an oven, and then desiccated with silica gel at room temperature until a constant weight was obtained (6–9 days). The experiments were repeated three times.

## Results and Discussion

The benzoxazinones, DIBOA-G, DIBOA, DIMBOA-G and DIMBOA, have been demonstrated to appear constitutively during the juvenile stage of growth and by *de novo* synthesis (Nakagawa *et al.*, 1995). Figure 2 shows their appearance and

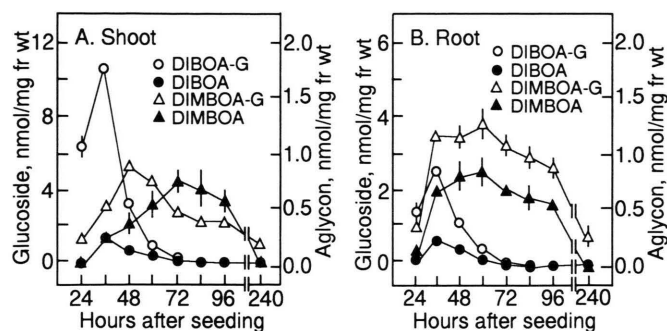


Fig. 2. Changes in contents of benzoxazinones. **A**, contents in shoot, and **B**, contents in root (reprinted from *Phytochemistry*, **38**, Nakagawa *et al.*, Non-induced occurrence of cyclic hydroxamic acids in wheat during juvenile stage of growth, 1349–1354 (1995) with kind permission from Elsevier Science Ltd., The Boulevard, Langford Lane, Kidlington OX5 1GB, UK).

disappearance in the shoot (**A**) and root (**B**) of the seedlings; the data are taken from the above literature.

#### UV spectra of benzoxazinone-ferric chloride complexes

Although ferric chloride reaction itself is not specific for benzoxazinones, their complexes are identified by the characteristic bluish color and absorption maximum (Hamilton, 1964). In methanol in the presence of ferric chloride, DIBOA-G gave a pale purple color with an absorption maximum at 550 nm, DIBOA a dark violet color with a maximum at 550 nm, DIMBOA-G a grayish blue color with a maximum at 580 nm, and DIMBOA a bright blue color with a maximum at 600 nm. The glucosides gave spectra with a blue shift when measured in aqueous methanol. Figure 3 shows the spectra of DIBOA-G with increasing amount of water, the absorption maximum being 540 nm and 530 nm in 2% and 5% aqueous methanol, respectively. Although data are not shown, DIMBOA-G gave a spectrum with a peak at 564 nm in methanol containing 5% water.

The methanol extract of shoots from 24-hr old seedlings whose benzoxazinone component is mainly DIBOA-G (Fig. 2) gave a spectrum with a peak at 530 nm (Fig. 3, d), corresponding to the authentic one measured in 5% aqueous methanol, and thus the water content of the methanol extract was considered to be about 5%. To confirm this, the plant material was desiccated to give a constant weight, and the water content of the fresh material was estimated to be  $88 \pm 3\%$ . Based on this, the water content of the methanol extract was calculated to be 4.4–4.5%. The extract gave a single purple spot corresponding to DIBOA-G when

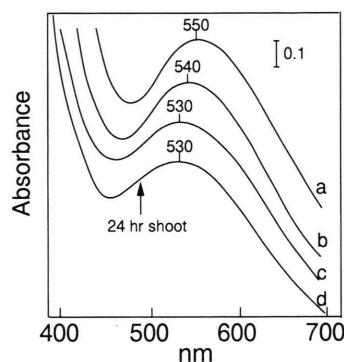


Fig. 3. UV absorption spectra of benzoxazinone-ferric chloride complexes. **a**, **b**, and **c**: DIBOA-G complex ( $3 \times 10^{-4}$  M) in methanol, methanol containing 2% and 5% water, respectively; **d**: methanol extract of shoots from 24-hr old seedlings treated with ferric chloride.

developed on a silica gel plate and sprayed with ferric chloride solution. This result showed that the blue component developed in the 24-hr-old seedlings is exclusively attributable to DIBOA-G. The extract of roots of 72-hr old seedlings that contain DIMBOA-G and DIMBOA in a ratio of 6:1 with little DIBOA-G and DIBOA (Fig. 2) had the characteristic blue color and gave a spectrum with a peak centered at 573 nm. Tlc of the extract gave two blue spots, the  $R_f$  values, 0.37 and 0.82, corresponding to those of DIMBOA-G and DIMBOA, respectively. No other blue spot was observed. These results made us possible to identify the localization in the tissues of benzoxazinones. Because of lability of benzoxazinones (Woodward *et al.*, 1978), we failed to prepare antigens by connecting them to albumin and thus antibodies, monoclonal or polyclonal.

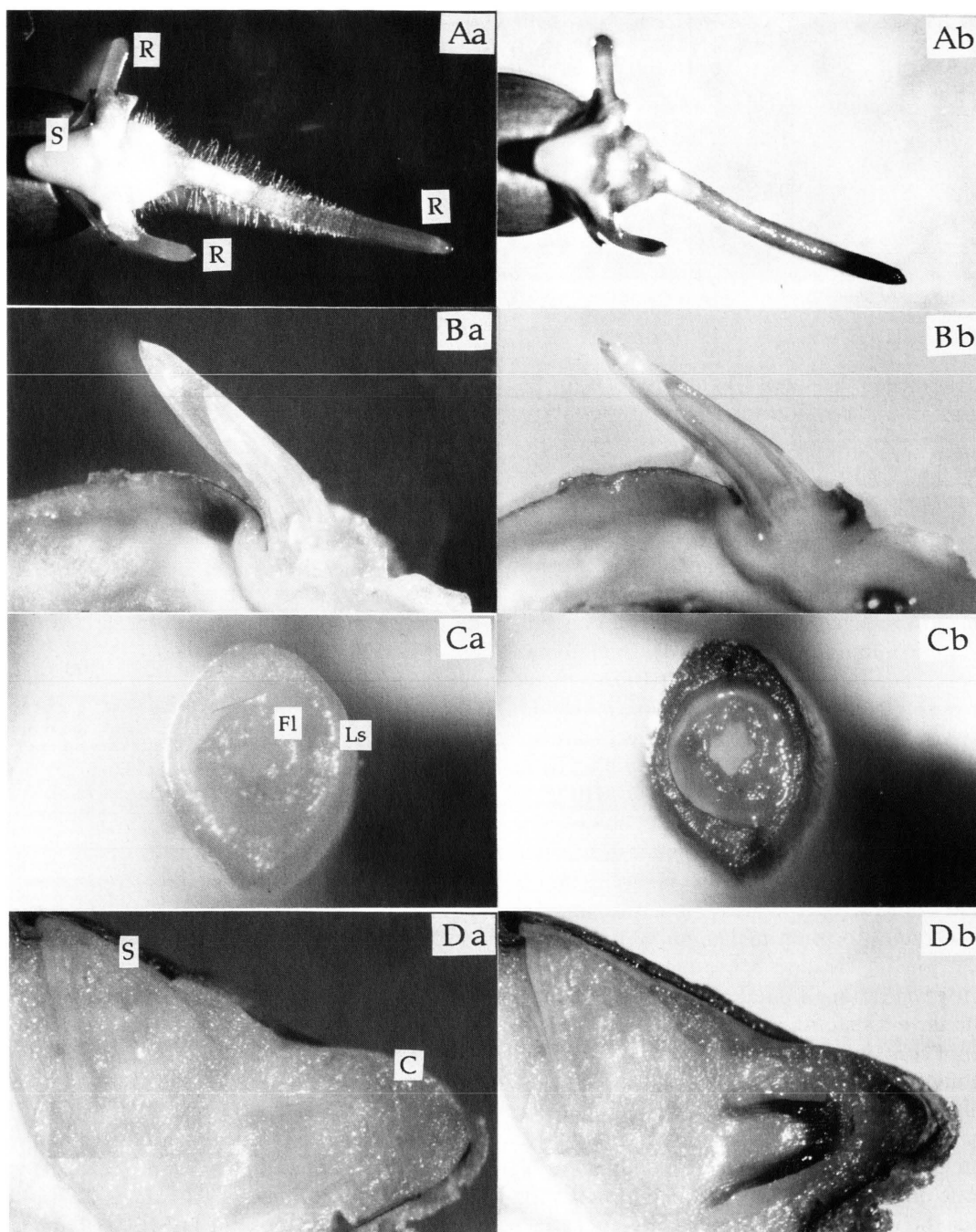


Fig. 4. **A:** Seedling grown for 32 hr after sowing; **a**, untreated and **b**, treated with ferric chloride solution. R denotes root, and S shoot. Magnification; x 10.5.  
**B:** Shoot of a 32-hr old seedling bisected longitudinally; **a**, untreated, and **b**, treated with ferric chloride solution. Magnification; x 21.  
**C:** Shoot of a 32-hr old seedling cut crosswise; **a**, untreated, and **b**, treated with ferric chloride solution. Ls denotes leaf sheath, and Fl foliage leaf. Magnification; x 52.5.  
**D:** Longitudinal section of embryo of a seed incubated for 18 hr after sowing; **a**, untreated, and **b**, treated with ferric chloride solution. S denotes shoot, and C emerging coleorhiza. Magnification; x 52.5.



### Stained seedlings

Figure 4A shows the stained and non-stained 32-hr old seedlings. In the roots, the color developed immediately after the application of the ferric chloride solution. The staining was strongest at the region close to the root cap and became lighter towards the basal part. The root cap was stained relatively less than the following region. The transverse sections of the stained root showed that the staining was stronger in the cortical part than in the inner part of the tissue, and the result was the same when the sections were made first and then stained. A histological specificity of the staining was not observed. These results seem to indicate that the occurrence of the benzoxazinones is prominent in the cortical part with rapidly dividing cells.

As shown in Fig. 4A, staining was not obvious when the ferric chloride solution was applied to the intact shoot, so that the shoot of the seedlings were cut into half and then stained. Figure 4B shows the results of a 32-hr old seedling. The color developed in both the sheath and leaf tissues, being stronger in the former than in the latter. Ferric chloride solution seems unable to permeate through the epidermal layer of the sheath. The staining was distributed rather uniformly through the tissue, and again the histological specificity in each organ was not observed. The same was also seen when the shoot was cut crosswise and stained (Fig. 4C). Although photographs are not shown, a similar result was obtained for older seedlings as well.

Coloring was not observed on the surface of the coleorhiza just emerged in a younger 18-hr old plant. To see the benzoxazinones in the radicle, the plant was cut into half along the longitudinal axis and stained. As Fig. 4D shows, staining occurred in the region corresponding to the cortex-epidermis complex of the radicle. The tip region corresponding to the quiescent, primordial root cap was stained relatively less, and the stele remained unstained. Staining was not observed in the leaf primordium, showing that benzoxazinones are synthesized faster in the radicle than in the

leaf primordium. Since the final step of the biosynthesis to produce DIBOA and DIMBOA has been reported to be the hydrolysis of the glucosides (Niemeyer, 1988) and the colored components come from DIBOA, DIMBOA and their glucosides, the final hydrolytic process is thought to occur within the stained region.

In the previous report (Nakagawa *et al.*, 1995), the concentrations in shoots of DIBOA and DIMBOA have been estimated based on HPLC analyses of the plant extracts to be 0.2–0.3 and 0.7–1.0 mM, respectively, assuming that the density of the plant tissue is unity and the distribution of the compound is uniform. The DIBOA and DIMBOA concentrations of 0.3 mM have been shown to be sufficient to retard the growth of the germ tube of pathogenic *Bipolaris sorokiniana*, non-pathogenic *Alternaria alternata*, and *Fusarium* spp. In the present study, these antimicrobial and antifeeding compounds and their immediate precursors, the glucosides, were found to occur in a concentrated manner in the cortical tissue of the roots and in the leaf sheath, the leaves folded inside the sheath being less stained. The situation may be more suitable or effective for preventing an attack by a pathogenic microbe or insect than their uniform distribution in the tissues. These results, together with the transient and limited appearance of the benzoxazinones during the juvenile stage of growth (Nakagawa *et al.*, 1995), suggest that the vulnerable seedlings utilize this process as a defense system, deepening our understanding on the biological significance of this novel class of compounds. The occurrence of benzoxazinones and its detection may be utilized as an index in hybridization experiments to produce cultivars resistant to attacks by deleterious microbes and insects.

### Acknowledgements

We express our sincere gratitude to Prof. Koichiro Tsunewaki, Department of Agricultural Biology, Faculty of Agriculture, Kyoto University, Kyoto, Japan, for providing seeds of *Triticum aestivum* cv Asakazekomugi. This work was supported in part by a Grant-in-Aid for Scientific Research (Project No. 03806021) from the Ministry of Education, Science and Culture of Japan.

- Argandoña, V. H., Niemeyer, H. M. and Corcuera, L. J. (1981), Effect of content and distribution of hydroxamic acids in wheat on infection by the aphid *Schizapis graminum*. *Phytochemistry* **20**, 673–676.
- Baker, E. A. and Smith, I. M. (1977), Antifungal compounds in winter wheat resistant and susceptible to *Septoria nodorum*. *Ann. Appl. Biol.* **87**, 67–73.
- Corcuera, J., Woodward, M. D., Helgeson, J. P., Kelman, A. and Upper, C. D. (1978), 2,4-Dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one, an inhibitor from *Zea mays* with differential activity against soft rotting *Erwinia* species. *Plant Physiol.* **61**, 791–795.
- Gutierrez, C., Castañera, P. and Torres, V. (1988), Wound-induced changes in DIMBOA (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one) concentration in maize plants caused by *Sesamia nonagrioides* (Lepidoptera: Noctuidae). *Ann. Appl. Biol.* **113**, 447–454.
- Hamilton, R. H. (1964), Tolerance of several grass species to 2-chloro-s-triazine herbicides in relation to degradation and content of benzoxazinone derivatives. *J. Agric. Food Chem.* **12**, 14–17.
- Lyons, P. H., Hipskind, J. D., Wood, K. V. and Nicholson, R. L. (1988), Separation and quantification of cyclic hydroxamic acids and related compounds by high-pressure liquid chromatography. *J. Agric. Food Chem.* **36**, 57–60.
- Nakagawa, E., Amano, T., Hirai, N. and Iwamura, H. (1995), Non-induced occurrence of cyclic hydroxamic acids in wheat during juvenile stage of growth. *Phytochemistry* **38**, 1349–1354.
- Niemeyer, H. M. (1988), Hydroxamic acids (4-hydroxy-1,4-benzoxazine-3-ones), defence chemicals in the Gramineae. *Phytochemistry* **27**, 3349–3358.
- Niemeyer, H. M., Pesel, E., Copaja, S. V., Bravo, H. R., Franke, S. and Francke, W. (1989), Changes in hydroxamic acid levels of wheat plants induced by aphid feeding. *Phytochemistry* **28**, 447–449.
- Woodward, M. D., Corcuera, L. J., Helgeson, J. P. and Upper, C. D. (1978), Decomposition of 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one in aqueous solutions. *Plant Physiol.* **61**, 796–802.
- Woodward, M. D., Corcuera, L. J., Helgeson, J. P., Kelman, A. and Upper, C. D. (1979), Quantitation of 1,4-benzoxazin-3-ones in maize by gas-liquid chromatography. *Plant Physiol.* **63**, 14–19.
- Wahlroos, Ö. and Virtanen, A. I. (1959), The precursors of 6-methoxybenzoxazolinone in maize and wheat plants, their isolation and some of their properties. *Acta Chem. Scand.* **13**, 1906–1908.