Pergamon

0031-9422(94)00831-0

Phytochemistry, Vol. 38, No. 6, pp. 1349 1354, 1995

0031 9422/95 $9.50 + 0.00

NON-INDUCED CYCLIC HYDROXAMIC ACIDS IN WHEAT DURING JUVENILE STAGE OF GROWTH

ERI NAKAGAWA, TAKASHI AMANO, NOBUHIRO HIRAI* and HAJIME IWAMURA†

Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan; *Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan

(*Received in revised form* 3 *October* 1994)

Key Word Index—*Triticum aestivum*; Gramineae; seedlings; hydroxamic acids; benzoxazinones; biosynthesis.

Abstract—2,4-Dihydroxy-1,4-benzoxazine-3-one glucoside (DIBOA-G) and its methoxy analogue, 2,4-dihydroxy-7-methoxy-1,4-benzoxazine-3-one glucoside (DIMBOA-G), were present in germinating wheat (*Triticum aestivum*); the corresponding aglycones, DIBOA and DIMBOA, appeared soon after germination. The amounts of these compounds reached a maximum 12–48 hours after germination, and then decreased to undetectable levels as the plants began autotrophic growth. The time of their appearance was little affected by using seeds either sterilized or non-sterilized, by infection with pathogens and wounding with a razor blade. The concentration of DIBOA was found to be 0.2–0.3 nmol mg^{-1} fr. wt (0.2–0.3 mM if the density of plant tissue is assumed to be uniform and unity) and that of DIMBOA was 0.7–1.0 nmol mg^{-1} (0.7–1.0 mM). The aglycones retarded the germ tube growth of species of fungi at 0.3 mM. These observations suggest that the appearance of benzoxazinones is as defence compounds in the juvenile stage of growth. [^{14}C]Anthranilic acid was incorporated into DIBOA-G and DIMBOA-G when administered to embryos isolated from pre-emerging seeds, showing that the series of compounds are generated by *de novo* synthesis.

INTRODUCTION

The cyclic hydroxamic acid, 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and its methoxy analogue, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), occur as glucosides in the Gramineae, including maize, wheat and rye [1, 2], and are implicated in the resistance of the plants to pathogens [2, 3] and insects [2, 4–7]. Most of the previous studies have been focused on autotrophic seedlings or adult plants, and, upon infection or by an insect attack, the glucosides are thought to be rapidly hydrolysed to produce DIBOA and DIMBOA [8–13]. The aglycones and their degradation products, 2-benzoxazolinone (BOA) and 6-methoxy-2-benzoxazolinone (MBOA), have been shown to exhibit fungistatic and bacteristatic activity, as well as antifeeding activity against insects, but the parent glucosides are essentially inactive [2, 11, 14].

We found that DIBOA-G and its methoxy analogue, DIMBOA-G, occur in germinating wheat, the aglycones, DIBOA and DIMBOA, soon after. The concentration of these compounds reached a maximum 12–48 hr after germination and then decreased to disappear as the plants grew to an autotrophic stage. This timing of appearance and disappearance was little affected by infections of pathogens, as well as by wounding plants with a razor blade. These findings suggested that the appearance of benzoxazinones is an event scheduled to occur in the early stage of growth.

†Author to whom correspondence should be addressed.

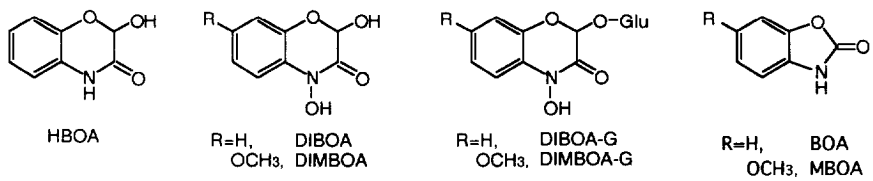
RESULTS

Extraction

Based on the stability of DIMBOA and DIBOA in aqueous solution [15], as well as recovery efficiencies of both the aglycones and glucosides [16], we chose weakly acidic 2% acetic acid–methanol as extraction solvent. The plant material to be extracted was immediately dipped in the alcoholic solvent at 50° to avoid enzymatic degradation of the glucosides, and extraction was made using an ultrasonic generator. The amounts of benzoxazinones extracted reached a plateau within 10 min and, under these condition, authentic DIBOA and DIMBOA suffered little degradation as well as their glucosides. Extraction with 2% acetic acid at 100° for 10 min was also examined but caused lowering of efficiency for recovery of DIMBOA, probably because of thermal decomposition.

HPLC analyses

To perform simultaneous analysis of hydroxamic acids, their glucosides and related benzoxazolinones, we examined the efficacy of a metal-free ODS column. A standard



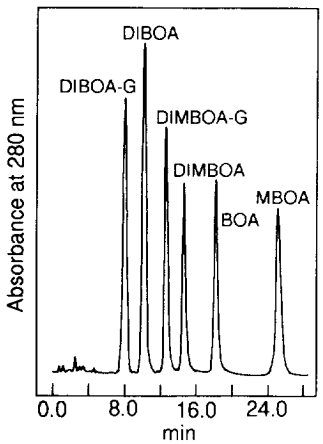


Fig. 1. HPLC chromatogram of cyclic hydroxamic acids, their glucosides and related benzoxazolinones. Separation was made using a metal-free C_{18} reversed-phase chromatography.

mixture of DIMBOA-G, DIBOA-G, DIMBOA, DIBOA, MBOA and BOA was resolved into six well-separated peaks (Fig. 1). The use of an ordinary ODS column gave sufficient separation of peaks of the glucosides as reported previously [16], but did not give base-line separation of the aglycones from their respective glucosides.

Occurrence of hydroxamic acids in seedlings

Wheat seeds germinated 20–24 hr after seeding under the incubation conditions used in this study. The results of the extraction and analyses are shown in Fig. 2. In germinating seeds, the concentration of DIBOA-G in the shoots was already very high (*ca* 6 nmol mg^{-1}fr. wt) (Fig. 2A). Its content reached a maximum 12–16 hr after germination (36 hr after seeding) and decreased rapidly thereafter. The aglycone, DIBOA, was detected soon after germination, reached a maximum (0.2–0.3 nmol mg^{-1} fr. wt, 0.2–0.3 mM assuming the density of the plant tissue to be uniform and unity, or *ca* 50 ppm) after 12 hr and then disappeared gradually. DIMBOA-G was found in the shoots of germinating seeds although its concentration was lower than that of DIBOA-G, and reached a maximum (5–6 nmol mg^{-1} fr. wt) *ca* 24 hr after germination (48 hr after seeding). DIMBOA appeared after germination and reached a maximum (0.7–1.0 nmol mg^{-1} fr. wt, 0.7–1.0 mM, or *ca* 200 ppm) 24 hr later than the glucoside, i.e. 72 hr after seeding. The contents of both DIMBOA-G and DIMBOA decreased gradually. The sequential appearance and disappearance of DIBOA-G, DIBOA and DIMBOA-G in roots (Fig. 2B) was similar to that in the shoots. DIMBOA appeared at a faster rate than that in shoots. Both aglycones were undetectable in fully autotrophic 10-day-old seedlings (240 hr after seeding). The glucoside DIBOA-G was undetectable, but DIMBOA-G was present at low levels. MBOA and BOA were not found in either shoots or roots during the experimental period.

Effects of sterilization and inoculation

To determine whether or not the appearance of DIBOA and DIMBOA is due to fungal or bacterial infection occurring during the experiments, we carried out experiments under sterile conditions. Seeds were dipped into 70% ethanol and then in 5% perchlorate. To examine the sterilization efficiency, seeds, their quarters and embryos were put on media for fungal and bacterial growth; sterilization was found to be perfect for 70–90% of the seeds for bacteria and 70–75% for fungi (Table 1). Based on these results, seeds were grown separately on a

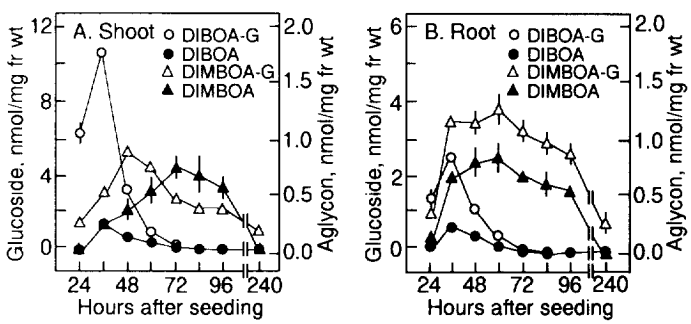


Fig. 2. Changes in contents of cyclic hydroxamic acids and their glucosides after germination. (A), Contents in shoots; (B), Contents in roots. (○ and ●) Contents of DIBOA-G and DIBOA, respectively; (△ and ▲,) contents of DIMBOA-G and DIMBOA, respectively. Vertical lines at data points show standard errors.

Table 1. Sterilization efficiency (%) of seeds against fungi and bacteria*

	Agar medium		
	Potato–sucrose	V8 juice	Yeast extract–peptone –glucose
Whole seeds	70	83	72
Quarters	85	90	70
Embryos	70	85	75

*Thrity to 40 seeds, their quarters and embryos were put on each medium and incubated for 4 days at 25°. Sterilization performance is expressed as percentage of cultures where no trace of fungal or bacterial growth was observed.

microplate to avoid infections from those individuals containing microbial contamination. The concentrations of the benzoxazinones were little different between experiments with non-sterilized and sterilized seeds, showing that their appearance is not due to infections (data not shown, but essentially the same as those shown in Fig. 2).

To confirm this finding, experiments were done using seeds inoculated with the pathogen *Bipolaris sorokiniana*, and strains of *Fusarium* and *Rhizopus* spp. so as to give severe symptoms after a culture of eight days. Again, no significant differences in the content of DIBOA and DIMBOA were observed between the experiments under infectious and non-infectious conditions. The effects of inoculation with non-pathogenic *Alternaria alternata* was also examined and the results obtained were the same.

Effects of wounding

No insects could enter into the container to injure the plants under the present experimental conditions. Nevertheless, we examined the possibility that wounding by a razor blade as a model of insect feeding induces the benzoxazinones. The apices of shoot and radicle of germinated seeds were cut off and then the plants grown for appropriate periods and extracted for HPLC analyses. No indications of induced occurrence of DIBOA or DIMBOA were observed (data not shown).

Antimicrobial activity of DIBOA and DIMBOA

Spores of *B. sorokiniana* and *A. alternata*, and microconidia of *Fusarium* spp were incubated in the presence of DIBOA and DIMBOA. No significant inhibition of germination was observed in the concentration range tested (0.03–3 mM), but inhibition of growth of germ tubes was notable at 0.3 mM (Table 2), the length of germ tube being on average 60% of that in the controls. Glucosides had little activity.

Occurrence of hydroxamic acids in seeds

Glucosides were already found to exist in the shoots of the germinated seeds (Fig. 2). We thus examined the occurrence and levels of these compounds in the seeds before germination. The glucosides were present 12–16 hr after seeding, while the aglycones were barely detectable before germination, up to 24 hr after seeding (Fig. 3).

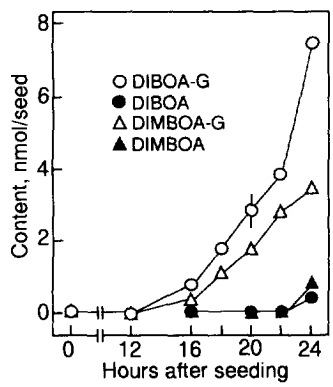


Fig. 3. Changes in cyclic hydroxaminic acid contents in pre-emergent seeds. (○ and ●), Contents of DIBOA-G and DIBOA, respectively; (△ and ▲), contents of DIMBOA-G and DIMBOA, respectively. Vertical lines at data points show standard errors.

Table 2. Inhibition of germ tube growth by benzoxazinones isolated from wheat*

		Germ tube length (μm)		
Compound	mM	*B. sorokiniana*	*A. alternata*	*Fusarium* spp.
None		50 ± 4.6† (100)‡	16.6 ± 1.52 (100)	8.2 ± 0.80 (100)
DIBOA	0.3	42 ± 3.8 (84)	9.0 ± 0.64 (54)	6.8 ± 0.42 (83)
	3	39 ± 3.2 (78)	10.0 ± 0.97 (60)	4.4 ± 0.38 (54)
DIMBOA	0.3	34 ± 2.6 (68)	13.6 ± 0.92 (82)	6.2 ± 0.38 (76)
	3	28 ± 1.9 (56)	11.2 ± 0.78 (65)	4.5 ± 0.33 (50)

*Spores of *B. sorokiniana* and *A. alternata* were incubated for 10 hr at 25 ± 1°, microconidia of *Fusarium* spp. for 24 hr.

†Standard error.

‡Values in parentheses are per cent of control.

Dry seeds were ground with a pestle and mortar and extracted as above, but no trace of aglycones or glucosides was found in the extracts.

Feeding of [^{14}C]anthranilic acid

A HPLC chromatogram of extracts of the shoots emerged from the embryos fed with [^{14}C]anthranilic acid is shown in Fig. 4. The results showed that label from anthranilic acid is incorporated into DIBOA-G and DIMBOA-G, indicating that the appearance of both compounds is by virtue of *de novo* synthesis. The peak in Fig. 4A that corresponds to fraction no. 6 in Fig. 4B with a high specific activity was identified as tryptophan. The specific activities of the DIBOA-G and DIMBOA-G isolated are shown in Table 3.

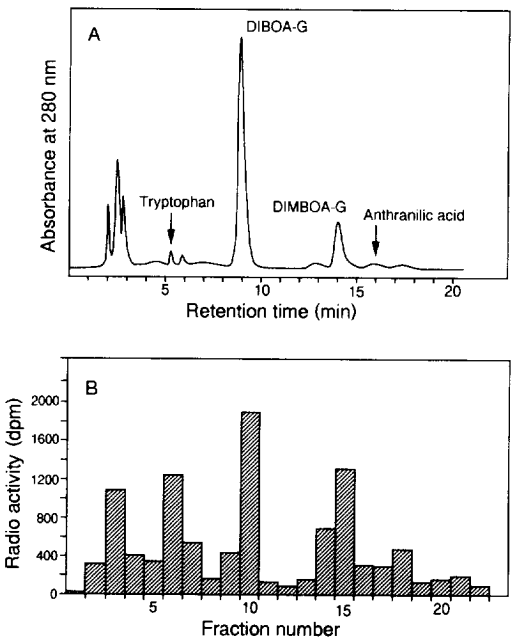


Fig. 4. HPLC chromatogram of extracts of shoots emerged from embryos fed with [^{14}C]anthranilic acid. (A), Chromatogram monitored by absorbance at 280 nm; (B), specific activity of chromatographic fractions.

Table 3. Incorporation of ^{14}C into DIBOA-G and DIMBOA-G from labelled anthranilic acid*

Compound	Amount (nmol)	Specific activity (Bq nmol^{-1})	Incoporation (%)†	Dilution
DIBOA-G	156.5	3.08	0.17	305
DIMBOA-G	28.5	13.8	0.14	68

*Thrity shoots cut out from embryos incubated with 300 μM [^{14}C] anthranilic acid for 18 hr were extracted and DIBOA-G and DIMBOA-G were isolated from extracts by HPLC.

†From [^{14}C]anthranilic acid.

DISCUSSION

DIMBOA and DIBOA are known to be heat-labile and transformed to their corresponding benzoxazolinones in neutral and alkaline media, and the glucosides present in plants are easily hydrolysed to give the aglycones upon standing homogenized plant material at room temperature. We thus extracted explants immediately with methanol containing 2% acetic acid at 50° in an ultrasonic generator, which gave an excellent recovery of the compounds causing little degradation. In the previous literature [4–6, 8, 17], benzoxazinones has been often estimated as DIBOA and/or DIMBOA equivalent after hydrolysis of the glucosides; this may have resulted in the failure to identify the occurrence of the aglycones in young plants [8].

The HPLC procedure with an ODS column [16] was satisfactory for the analysis of a mixture of glucosides, but not for the analysis of a mixture of both glucosides and aglycones, giving overlapping peaks of the glucoside and its aglycone without base-line separation. Since hydroxamic acids function as a metal chelator, this property may disturb their separation on an ordinary ODS column. We used a metal-free column which gave an excellent separation of the peaks of the glucosides from their aglycones (Fig. 1).

The extraction and analysis were done on shoots and roots of wheat seedlings under non-autotrophic to early autotrophic growth, i.e. juvenile stage (Fig. 2). The development of benzoxazinones was little affected by the inoculation of either pathogenic or non-pathogenic fungi or wounding. These findings suggest that these compounds, especially the aglycones DIMBOA and DIBOA, appeared not as phytoalexins but according to a schedule.

A number of previous studies have suggested the role of hydroxamic acids in the resistance of Gramineae plants to pathogens and insects [2, 4, 8, 17–19]. We have shown here that DIBOA and DIMBOA retarded the growth of the germ tubes of *B. sorokiniana*, *A. alternata* and *Fusarium* spp at 0.3 mM (Table 2). The concentration of DIBOA in plants was found to be $\sim$0.3 nmol mg^{-1} fr. wt or 0.3 mM and that of DIMBOA was $\sim$1 nmol mg^{-1} fr. wt or 1 mM (Fig. 2), assuming that the compounds are uniformly distributed. Actually, however, the aglycones may be localized more in cortical tissues than in internal tissues to prevent attacks by pathogenic microbes and insects. The activation or induction of glucosidases is thought to occur little since the levels of DIBOA and DIMBOA changed little between the experiments under infectious and sterilized conditions.

The glucosides started to appear in seeds in the pre-emergence stage and the aglycones formed concurrently with germination (Fig. 3). Hydroxamic acids have already been shown to be absent from dry seeds [2, 8]. However, to examine whether or not DIBOA and DIMBOA are stored in the form of glucosides or their high M_r conjugates,

dry seeds were ground and exhaustively extracted with methanol; no traces of benzoxazinones or their glycosides were found. The results were the same when ground and macerated dry seeds were treated with hydrolytic enzymes, such as β-glucosidase, α-amylase, cellulase, pectinase, β-galactosidase, pronase and esterase (data not shown). We then fed embryos isolated from pre-emerging seeds with labelled anthranilic acid, a compound on the shikimic acid pathway from which benzoxazinones are derived [20, 21]. Isotope was effectively incorporated into DIBOA-G and DIMBOA-G (Fig. 4). The results indicate that the generation of the benzoxazinones is by virtue of *de novo* synthesis.

DIMBOA-G has been reported to be biosynthesized from 2-hydroxy-1,4-benzoxazin-3-one (HBOA), hydroxylation at the 7-position and methylation occurring on this compound or its glucoside, rather than on DIBOA or DIBOA-G [21]. DIBOA-G is also biosynthesized from HBOA but without hydroxylation and label from HBOA is more rapidly incorporated into DIBOA than into DIMBOA [2, 21]. In our experiments, dilution after 18 hr of incubation with [^{14}C]anthranilic acid was higher in DIBOA-G than in DIMBOA-G. DIBOA-G is biosynthesized more efficiently than DIMBOA-G (Fig. 3) and, thus, labelled DIBOA-G could be diluted more efficiently by non-labelled compound than DIMBOA-G under conditions where the supply of labelled precursor is thought to be limited. The present results lead us to suppose that plants at a vulnerable, juvenile stage may utilize this process to act as a defence against attacks by pathogens and insects.

EXPERIMENTAL

Preparation of DIBOA-G, DIMBOA-G and DIBOA from wheat seedlings. Shoots of 11 000 seedlings grown for 40 hr were extracted with 350 ml of hot 2% HOAc for 10 min. The soln was filtered and extracted with *n*-BuOH (×4) and the organic layers combined and concd under red. pres. The residue was chromatographed on a silica gel column that was eluted with $CHCl_3$ containing MeOH and 1% HOAc. The frs eluted with 3 and 10% MeOH were combined and purified by HPLC using a 1 cm i.d. × 25 cm A-323 ODS column (YMC Co.) that was eluted with 0.3% HOAc–30% MeOH–H_2O at a flow rate of 3 ml min^{-1} with monitoring at 254 nm. The yields of DIBOA, DIBOA-G and DIMBOA-G were 14, 13 and 9 mg, respectively. The compounds were identified by ^{1}H NMR, MS and UV spectra [15,16].

Preparation of DIMBOA, MBOA and BOA. DIMBOA. A 4.4 mg aliquot of DIMBOA-G was dissolved in 0.5 ml of HOAc–NaOAc buffer (100 mM, pH 5.1) to which 5 mg of β-glucosidase (22 units) (Sigma) was added. The mixt. was incubated at 37° for 4 hr, diluted to 6 ml with distilled H_2O, made acidic with 1 M HCl and extracted with EtOAc. The organic layer was evapd under red. pres. to dryness giving 2.6 mg of DIMBOA. *MBOA.* A 1.7 mg aliquot of DIMBOA was dissolved in 2 ml Pi buffer (10 mM, pH 7.5) and the mixt. heated at 80° for 1 hr. The reaction mixt. was made acidic, diluted to 6 ml with distilled H_2O and extracted with EtOAc. The organic layer was evapd under red. pres. to dryness giving 1 mg of residue. The residue was chromatographed on a HPLC system equipped with a 6 mm i.d. × 10 cm AQ-311 ODS column (YMC Co.) that was eluted with 0.1% HOAc–35% MeOH–H_2O at a flow rate of 1 ml min^{-1} with monitoring at 280 nm to give 0.7 mg of MBOA. *BOA.* A 3.3 mg aliquot of DIBOA was dissolved in 3 ml of Pi buffer (10 mM, pH 7.5) and the soln heated at 80° for 1 hr. The mixt. was treated as described above for the prepn of MBOA and the collection of the major UV-absorbing peak on the HPLC chromatogram gave 1.8 mg of BOA.

Analysis of benzoxazinones and related compounds in plant material by HPLC. Seedlings were divided into parts which were immediately ultrasonicated with 2% HOAc–MeOH at 50° for 10 min. The mixt. was filtered and 25 ml of the filtrate was injected into a 6 mm i.d. × 10 cm metal-free Wakosil-II-5C18-HG column (Wako) which was then eluted with 0.1% HOAc–22% MeOH–H_2O at 40° at a flow rate of 1 ml min^{-1}. Monitoring was made at 280 nm for the analyses of benzoxazinones and benzoxazolinones, and at 330 nm for anthranilic acid. Dry seeds were ground in a mortar with a pestle and then extracted and analysed as described above.

Seedling culture. Wheat seeds (50) were placed in a Petri dish (9 cm i.d. × 1.5 cm height) that contained three layers of filter paper and 20 ml distilled H_2O. The seeds were incubated at 25° with a 12-hr period of illumination from fluorescent lamps.

Sterilization of seeds and their culture. Seeds were soaked in 70% EtOH for 1 min, washed several times with distilled H_2O, immersed in 5% $NaClO_4$ soln for 10 min and washed thoroughly. Individual seeds were put into a hole of a microplate (Corning Cell Wells) and incubated under sterile conditions at 25° with a 12-hr period of illumination from fluorescent lamps. Sterilization performance was examined as follows. Sterilized seeds, those divided into quarters and embryos were put on a potato–sucrose–agar medium and also on a 5% V8 juice-agar medium to examine growth of fungi. For the detection of bacteria, Nissui standard method agar 05618 containing yeast extract, peptone, glucose and agar was used. Incubation was made at 25° for 4 days and sterilization performance was expressed by percentages of the cultures where any trace of fungal or bacterial growth was not observed.

Inoculation of seeds. Strains of *Bipolaris sorokiniana* (Saccarolo in Sorokin) Shoemaker that cause brown spots on Gramineae plants and non-pathogenic *Alternaria alternata* (Fries) Keissler were cultured on V8-agar medium at 25° for 10 days. Spores obtained by washing the surface of the agar medium were suspended in 50 ml of distilled H_2O in which seeds were soaked for 15 min at room temp. Spores of strains of *Fusarium* and *Rhizopus* isolated from seedlings infected in a Petri dish were grown on a potato–sucrose–agar medium and inoculation was made as described above.

Wounding of seedlings. Apices of shoots and radicles of seedlings grown for 30 hr were cut off with a razor blade and the rest of the plant was grown in a Petri dish as described above.

Antimicrobial assay. An appropriate concn of test compound in MeOH was put into a hole slide glass (1.4–1.5 cm i.d. × 0.6 mm depth) and dried under a stream of air and then in a desiccator under red. pres. Spores of *A. alternata* and *B. sorokiniana*, and microconidia of *Fusarium* spp., were suspended in 10 mM Pi buffer (pH 6) [17], filtered through two layers of gauze and diluted to a density of 10^3 cells ml^{-1}. A 0.15 ml aliquot of the suspension was dropped into the hole of a slide glass and incubated at 25 ± 1° in a chamber moistened with wet filter paper. The per cent of germination and the germ tube length were measured after 10 hr for *A. alternata* and *B. sorokiniana*, and after 24 hr for *Fusarium* spp.

Feeding with [^{14}C]anthranilic acid. Embryos (50) cut out from seeds incubated with distilled H_2O at 25° for 6 hr were placed in a Petri dish (4 cm i.d. × 1 cm depth) containing 1 ml of 300 μM [ring-UL-^{14}C]anthranilic acid (sp. act. 940 MBq $mmol^{-1}$, Sigma) and incubated at 25° for 18 hr under continuous illumination. Shoots were cut out from 30 germinated embryos and ultrasonicated with 1 ml of 2% HOAc in MeOH at 50° for 10 min. The extracts were filtered, evapd to dryness under a stream of Ar and dissolved in 100 μl of 30% MeOH containing 0.1% HOAc. A 10 μl aliquot of the soln was subjected to HPLC as described above and 30 frs of 1 ml each were collected. The rest, 90 μl of extract, was subjected to HPLC and compounds eluted after 9.6 and 14.7 min were collected to give DIBOA-G and DIMBOA-G. Both compounds were purified by HPLC to constant sp. act.

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 aestavum* var. Asakazekomugi, and to Prof. Mitsuya Tsuda (Pesticide Research Institute, Faculty of Agriculture, Kyoto University, Kyoto, Japan) for his gift of strains of *Bipolaris sorokiniana* and *Alternaria alternata*. 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.

REFERENCES

1. BeMiller, J. N. and Pappelis, A. J. (1965) *Phytopathology* **55**, 1241.
2. Niemeyer, H. M. (1988) *Phytochemistry* **27**, 3349.
3. Wahlroos, O. and Virtanen, A. I. (1958) *Acta Chem. Scand.* **12**, 124.
4. Argandoña, V. H., Luza, J. G., Niemeyer, H. M. and Corcuera (1980) *Phytochemistry* **19**, 1665.
5. Gutierrez, C., Castañera, P. and Torres, V. (1988) *Ann. Appl. Biol.* **113**, 447.
6. Niemeyer, H. M., Pesel, E., Copaja, S. V., Bravo, H. R., Franke, S. and Francke, W. (1989) *Phytochemistry* **28**, 447.
7. Scriber, J. M., Tinguy, W. M., Gracen, V. E. and Sullivan, S. C. (1975) *J. Econ. Entomol.* **68**, 823.
8. Argandoña, V. H., Niemeyer, H. M. and Corcuera, L. J. (1981) *Phytochemistry* **20**, 673.
9. Hofman, J. and Hofmanová, O. (1971) *Phytochemistry* **10**, 1441.
10. Scism, A. J., BeMiller, J. N. and Caskey, A. L. (1974) *Anal. Biochem.* **58**, 1.
11. Wahlroos, O., and Virtanen, A. I. (1959) *Acta Chem. Scand.* **13**, 1906.
12. Wahlroos, O, and Virtanen, A. I. (1964) *J. Pharm. Sci.* **53**, 844.
13. Willard, J. I. and Penner, D. (1976) *Residue Rev.* **64**, 67.
14. Baker, E. A. and Smith, I. M. (1977) *Ann. Appl. Biol.* **87**, 67.
15. Woodward, M. D., Corcuera, L. J., Helgeson, J. P. and Upper, C. D., (1978) *Plant Physiol.* **61**, 796.
16. Lyons, P. C., Hipskind, J. D., Wood, K. V. and Nicholson, R. L. (1988) *J. Agric. Food Chem.* **36**, 57.
17. Corcuera, L. J., Woodward, M. D., Helgeson, J. P., Kelman, A. and Upper, C. D. (1978) *Plant Physiol.* **61**, 791.
18. Couture, R. M., Routley, D. G. and Dunn, G. M. (1971) *Physiol. Pl. Path.* **1**, 515.
19. Sahi, S. V., Chilton, M.-D. and Chilton, W. S. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3879.
20. Reimann, J. E. and Byerrum, R. W. (1964) *Biochemistry* **3**, 847.
21. Tipton, C. L., Wang, M.-C., Tsao, F. H.-C., Tu, C. L. and Husted, R. R. (1973) *Phytochemistry* **12**, 347.