PN-ABD-576
63 7.404

Research Report No. 2

Grant: DPE-5542-G-SS-8077-00

Period covered:

From: March 1, 1989

To: August 31, 1989

Preliminary remarks

In spite of having submitted a "Request for Advance or Reimbursement" for the present reporting period in April 25, no further funds have been received, as of this date, corresponding to the present reporting period. It is regretable that this has lead to delays in the payment of salaries to students and technicians and in the purchase of supplies. It is hoped that provision of funds will be regularized in the future.

Wheat-aphid-virus interactions mediated by hydroxamic acids (Hx)

Choice experiments

Leaves of two wheat cultivars in the 1-leaf stage growing in pots were placed in a clip cage and 4 apterous aphid adults were introduced. Six hours later the distribution of aphids in the cage was recorded. Each experiment consisted of 22 clip cages. Duplicate or triplicate experiments were performed. Results are shown in Table 1.

Rec'd in SCI OCT 17 1989

Table 1. Choice experiments[a]

DIMBOA level[b]	Low 0.9	Low 1.0	Medium 1.3	Medium 1.4	High 2.0	High 2.7
Cultivar	Platifen	Millaleu	Mexifen	Nobo	Anza	Maiten
Platifen	39:38	34:26	43:36	33:28	53:27[c]	42:21[c]
Millaleu		35:40	39:29	38:32	56:24[c]	42:21[c]
Mexifen			36:36	38:38	39:28	57:18[c]
Nobo				36:31	44:28[c]	44:31[c]
Anza					28:28	37:40
Maiten						21:23

[a] Figure to the left of the colon refers to the cultivar at the left. Figure to the right of the colon refers to the cultivar above.
[b] Values in mmol/kg fr. wt.
[c] Differences in distribution are significant [$p(X^2)<0.1$].

A tendency is clear for aphids to settle preferentially on those leaves containing lower DIMBOA levels.

Electropenetration graphs

The system used was that developed by Tjallingii [Aphids: Their Biology, Natural Enemies and Control, Minks, A.K. and Harrewijn, P., eds., 1988, p. 95]. Waves were identified corresponding to the following activities: probing, sieve element ingestion and xylem ingestion. The behavior of twenty adult apterous aphids was observed for each cultivar studied. The results are shown in Table 2.

Table 2. Parameters from electropenetration graphs of aphid feeding activities[a]

DIMBOA level	Low		Medium		High	
Cultivar	Platifen	Millaleu	Mexifen	Nobo	Anza	Maiten
Time to penetration into sieve elements	20.7 a (3.2)	29.4 a (3.9)	61.8 b (6.3)	64.5 b (4.3)	88.4 c (7.2)	124.5 d (5.6)
Time to sieve element ingestion	55.0 a (4.0)	55.4 a (6.4)	67.1 b (6.5)	71.3 b (7.6)	95.0 c (7.3)	153.2 d (4.9)
Time in xylem ingestion	34.3 a (3.4)	35.0 a (3.3)	50.1 b (6.5)	50.5 b (3.3)	77.1 c (6.9)	83.0 c (4.5)
% arriving at sieve element	95	85	50	50	30	50

[a] Values followed by the same letter within a row are not significantly different [$p(t)<0.05$]. Values in parenthesis are standard errors.

The results show that higher DIMBOA levels lead to fewer aphids reaching the phloem. As the DIMBOA levels in plants increased, time to penetration to and committed ingestion in sieve elements increased, and time spent in ingestion from xylem also increased.

BYDV titration of infested wheat plants

The different wheat lines used in the studies above were exposed to aphids which were reared on BYDV infected oat. For each cultivar studied, twenty plants with one aphid each in a

clip cage were used. After 6 hours aphids were changed to oats and were left there for 24 hours. Both wheat and oat plants were then sprayed with insecticide (Dimethoate) and further left to grow for 30 days. After thus period they were assayed by ELISA method for BYDV titre. The results are shown in Table 3.

Table 3. BYDV titre and percentage of infected plants by viruliferous aphids on tested wheats

	Platifén	Millaleu	Mexifén	Nobo	Anza	Maiten
BYDV Titre	0.42a* (0.18)	0.42a (0.12)	0.34a (0.08)	0.30b (0.06)	0.27c (0.07)	0.28c (0.06)
% of infected plants	80	71	80	78	22	50

* Values followed by same letter are not significantly different ($p < 0.05$, Student's t-test).

These results clearly suggest that Hx play an important role in BYDV infection of wheat, presumably through antifeedant effects on the aphids vectors.

Toxicity and reactivity of hydroxamic acids

The reaction of DIMBOA, the main Hx in wheat, with α-chymotrypsin, a proteolytic enzyme, was described. It was shown that DIMBOA reacted with the hyper reactive serine moiety in the active site through the α,β-dicarbonyl system of its aldol form. The results are summarized in the accompanying manuscript (submitted to Phytochemistry). These model studies have been

used in the interpretation of the effect of DIMBOA on phytophagous pest larvae on maize, another DIMBOA-containing cereal.

Hydroxamic acid levels in wheat and its relatives

Preliminary results with Hx analysis of perennial Triticeae have shown that:

a) Hx could be detected in *Hordeum* species; previous analysis of numerous cultivated *Hordeum* accessions had not shown the presence of these compounds;

b) Wild *Secale* accessions showed only DIBOA (the demethoxylated analogue of DIMBOA), in agreement with previous analysis of cultivated rye.

Exploitation of allelopathic effects of hydroxamic acids

Wild oat, *Avena fatua* L., is one of the most troublesome annual grass weeds of wheat and other cereals. Infestation by wild oat causes extensive cereal crop yield losses. Numerous pre and postemergence herbicides have been developed for the control of wild oat in various crops. An alternative way to control weeds in crop is through allelochemicals that inhibit the germination and growth of weed species, and are produced and released by growing crop plants or their residues. This alternative for weed control is receiving increased attention due to its potential in avoiding adverse environmental effects.

Recently, DIBOA, the Hx isolated from rye, and its decomposition product benzoxazolin-2-one, were associated with

the allelopathic effects exhibited by this crop [Barnes and Putnam (1987) J. Chem. Ecol. 13, 889]. The phytotoxic effect of DIMBOA and its decomposition product MBOA on *A. fatua*, a worldwide weed affecting wheat, and on *A. sativa*, a crop normally rotated with it, were studied. The results are described in the accompanying manuscript (accepted in Phytochemistry).

Effects of Hx on aphid parasitoids and predators

Although biological control and host plant resistance are often compatible and even complementary, variations in host insects, in particular through variations in their host plants, may have dramatic effects on their predators. Since we have found DIMBOA in aphids that have fed on wheat plants, it is considered necessary to study the effects of DIMBOA on aphid predators. One of the most important aphid predators in Chile is the coccinelid *Eriopis connexa*.

A colony of *E. connexa* is being established using aphids as food source. Additionally, a diet is being developed in order to test compounds directly.

Summary of publications

1. Pérez, F.J. (1990) Allelopathic effect of hydroxamic acids from cereals on oat *Avena sativa* L. and wild oat *A. fatua* L.. *Phytochemistry*, in press.

2. Cuevas, L., Niemeyer, H.M, and Pérez, F.J. (1990) Reaction of DIMBOA, a resistance factor from cereals, with α-chymotrypsin. *Phytochemistry*, submitted.

Summary of presentations at international meetings

1. Niemeyer, H.M. and Givovich, A. (1989) The role of hydroxamic acids from wheat in pest and disease resistance. **Seventh International Symposium on Insect-Plant Relationships**. Budapest, Hungary, July 3-8.

2. Niemeyer, H.M., Pérez, F.J., and Cuevas, L. (1989) Chemical basis for toxicityof hydroxamic acids from cereals. **32nd IUPAC Congress**, Stockholm, Sweden, August 2-7.

3. Niemeyer, H.M., Givovich, A. and Copaja, S.V. (1989) Chemical basis of wheat defense against pests and diseases. **Annual Meeting of the International Society of Chemical Ecology**, Gothemburg, Sweden, August 7-11.

FEDERAL CASH ADVANCE STATUS REPORT
(Report Control No. W-245)

Grant: DPE-5542-G-SS-8077-00

A. Period to be covered by this report:
From: March 1, 1980
To: August 31, 1989

Period covered by next report:
From: September 1, 1989
To: February 28, 1990

B. Cash Advance Use and Needs:

1. Cash advance on hand at the beginning of this reporting period US$ 31,763.82

2. U.S. Treasury check advance received during this reporting period US$ 0.00

3. Interest earned on cash advance during this reporting period US $ 0.00

4. GROSS cash advance available during this reporting period US $ 31,763.82

5. LESS interest remitted to AID during this reporting period US $ 0.00

6. NET cash advance available during this reporting period US $ 31,763.82

7. Total disbursements during this reporting period, including subadvances US $ 31,763.82

8. Amount of cash advances available at the end of this reporting period US $ 0.00

9. Projected disbursements, including subadvances, for the next reporting period US $ 36,100.00

10. Additional cash advance requested for the next reporting period US $ 36.100.00

11. Total interest earned on cash advance from the start of the grant to the end of this reporting period, but not remitted to AID US $ 0.00

12. Total cash advances to subgrantees, if any, as of the end of this reporting period US $ 0.00

8'

C. Certification

The undersigned hereby certifies: (1) that the amount in paragraph B.9 above represents the best estimate of funds needed for the disbursements to be incurred over the period described, (2) that appropriate refund or credit to the grant will be made in the event of disallowance in accordance with the terms of the grant, (3) that appropriate refund or credit to the grant will be made in the event funds are not expended, and (4) that any interest accrued on the funds made available herein will be refunded to AID.

FACULTAD DE CIENCIAS
UNIVERSIDAD DE CHILE
Oficina Central de Importaciones y
Proyectos de Investigación

September 30, 1989

Juan Carrasco
Project Officer
Universidad de Chile

D. Summary of purchases made during reporting period

Beckman Instruments	Isotope detector	US$ 16,074.00
Beckman Instruments	Difference in freight	972.36
Sigma Chemical Co.	Chemicals	515.10
Local suppliers	Laboratory supplies	4,013.19
H.M. Niemeyer	Travel funds	7,870.00
Students, technicians	Salaries	2,319.17
	TOTAL	US$ 31,763.82

REQUEST FOR ADVANCE OR REIMBURSEMENT

(See instructions on back)

Approved by Office of Management and Budget, No. 80-R0183

PAGE 1 OF 1 PAGES

1. TYPE OF PAYMENT REQUESTED
a. "X" one, or both boxes: [X] ADVANCE [ ] REIMBURSEMENT
b. "X" the applicable box: [ ] FINAL [X] PARTIAL

2. BASIS OF REQUEST: [X] CASH [ ] ACCRUAL

3. FEDERAL SPONSORING AGENCY AND ORGANIZATIONAL ELEMENT TO WHICH THIS REPORT IS SUBMITTED: Agency for International Development

4. FEDERAL GRANT OR OTHER IDENTIFYING NUMBER ASSIGNED BY FEDERAL AGENCY: DPE-5542-G-SS-8077-00

5. PARTIAL PAYMENT REQUEST NUMBER FOR THIS REQUEST: 2

6. EMPLOYER IDENTIFICATION NUMBER:

7. RECIPIENT'S ACCOUNT NUMBER OR IDENTIFYING NUMBER: AID-Niemeyer

8. PERIOD COVERED BY THIS REQUEST
FROM (month, day, year): March 1, 1989
TO (month, day, year): February 28, 1990

9. RECIPIENT ORGANIZATION
Name: Universidad de Chile
Number and Street: Banco de Chile, NY Branch
Acc. No. 200022-02
124 East 55th St.
City, State and ZIP Code: NY, NY 10022

10. PAYEE (Where check is to be sent is different than item 9)
Name:
Number and Street:
City, State and ZIP Code:

11. COMPUTATION OF AMOUNT OF REIMBURSEMENTS/ADVANCES REQUESTED

PROGRAMS/FUNCTIONS/ACTIVITIES ▶	(a)	(b)	(c)	TOTAL
a. Total program outlays to date (As of date)	$	$	$	$
b. Less: Cumulative program income				
c. Net program outlays (Line a minus line b)				
d. Estimated net cash outlays for advance period				
e. Total (Sum of lines c & d)				
f. Non-Federal share of amount on line e				
g. Federal share of amount on line e				
h. Federal payments previously requested				
i. Federal share now requested (Line g minus line h)				
j. Advances required by month, when requested by Federal grantor agency for use in making prescheduled advances — 1st month				
2nd month				
3rd month				

12. ALTERNATE COMPUTATION FOR ADVANCES ONLY

a. Estimated Federal cash outlays that will be made during period covered by the advance	$ 36,100.-
b. Less: Estimated balance of Federal cash on hand as of beginning of advance period	0
c. Amount requested (Line a minus line b)	$ 36,100.-

13. CERTIFICATION

I certify that to the best of my knowledge and belief the data above are correct and that all outlays were made in accordance with the grant conditions or other agreement and that payment is due and has not been previously requested.

SIGNATURE OF AUTHORIZED CERTIFYING OFFICIAL: [signature]

TYPED OR PRINTED NAME AND TITLE: Dr. Camilo Quezada, Dean

DATE REQUEST SUBMITTED: October 2, 198

TELEPHONE (AREA CODE, NUMBER, EXTENSION): (2) 2712865 ext. 206

This space for agency use

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REACTION OF DIMBOA, A RESISTANCE FACTOR FROM CEREALS, WITH α-CHYMOTRYPSIN

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Key Word Index: Benzoxazinones, hydroxamic acids, DIMBOA, Gramineae, plant defence, α-chymotrypsin

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Abstract 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), a hydroxamic acid involved in pest resistance of cereals, inactivated α-chymotrypsin (E.C.4.21.2). Semilog plots of the residual activity of α-chymotrypsin under an excess of DIMBOA as a function of time were not linear. A kinetic model which considers the spontaneous decomposition of DIMBOA provided a quantitative account for these results. N-Acetyl-L-phenylalanine ethyl ester protected the enzyme against DIMBOA inactivation. Aminoacid analysis showed a significant decrease in lysine residues in the modified enzyme as compared with the native one. The loss of enzyme activity by reaction with DIMBOA was simultaneous with the decrease in titre of active site serine. These results suggested reaction of DIMBOA with the active site serine residue.

The DIMBOA analogue 4-hydroxy-2,7-dimethoxy-1,4-benzoxazin-3-one showed no effect on the enzyme. This result suggested that the reaction of DIMBOA with α-chymotrypsin occurred with the participation of the aldol tautomer of DIMBOA.

INTRODUCTION

2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA, 1), the main hydroxamic acid (Hx) isolated from extracts of maize and other cereals [1], has been involved as a resistance factor toward the European corn borer *Ostrinia nubilalis*, a major pest of corn [2]. When larvae of *O. nubilalis* are fed with diets containing DIMBOA at concentrations higher than 0.4 mg DIMBOA/g diet, consumption increased, a behaviour interpreted as an attempt of the larvae to compensate for antinutritional effects of DIMBOA [2].

Gut extracts of *O. nubilalis* showed proteinase activities toward different synthetic substrates. Highest specific activities were obtained with N-benzoyl arginine ethyl ester (BAEE) and with N-benzoyl tyrosine ethyl ester (BTEE). DIMBOA inhibited these activities *in vivo* and *in vitro*. The major inhibitions were obtained when BAEE and BTEE were used as substrates [F. Campos, J. Houseman, J. Atkinson and J.T. Arnason, personal communication], suggesting that the main proteinase activities in the gut extracts affected by DIMBOA are tryptic and chymotryptic in nature.

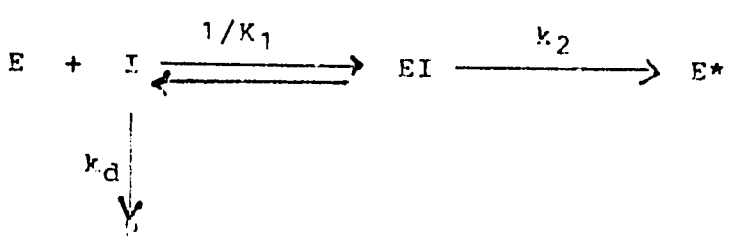
Since enzymes from insects are, in general, comparable to the corresponding enzymes from vertebrates [3], we studied the reaction of DIMBOA with well-known bovine α-chymotrypsin, as a model for that of *O. nubilalis*.

RESULTS AND DISCUSSION

Kinetic analysis

Semilog plots of the decrease with time of α-chymotrypsin activity under an excess of DIMBOA at pH 7.5 were not linear. After 8 h of reaction, complete inactivation was not obtained at every DIMBOA concentration studied and the curves describing the inactivation process were asymptotic with time (Fig. 1). Several models in which the inactivator partially modifies the catalytic activity of the enzyme may account for the results [4]. However, DIMBOA decomposes in aqueous solution with first order kinetics, the decomposition rate at pH 7.5 and 30° being 8×10^{-2} min^{-1} [5]. Hence, after 8 h of reaction only 2% of the initial DIMBOA concentration remains in solution. A kinetic model for the inactivation of an enzyme by an unstable inactivator that decomposes spontaneously with first order kinetics has been described [6-9]. The model may be represented by the scheme below, in which k_d is the unimolecular decomposition rate of the inhibitor and E* is the inactive enzyme.

Scheme 1



$$\ln \frac{E}{E_o} = \frac{k_2}{k_d} \ln \frac{K_1 + (I)_o e^{-k_d t}}{K_1 + (I)_o} \qquad (1)$$

Two approaches were employed for the determination of the kinetic constants in this model: analysis of the time dependence of the inactivation process, and analysis of the dependence of the end point of the enzyme inactivation on the initial inactivator concentration. For this latter procedure equation 1 was transformed to equation 3 by setting $t = \infty$ and establishing the condition $(I)_o >> K_1$.

$$\log \frac{E}{E'_o} = \frac{k_2}{k_d} \log \frac{K_1}{K_1 + (I)_o} \qquad (2)$$

$$\log \frac{E}{E_o} = - \frac{k_2}{k_d} \log (I)_0 + \frac{k_2}{k_d} \log K_1 \qquad (3)$$

The experimental points for the time dependence inactivation of α-chymotrypsin at different DIMBOA concentrations were least-squares fitted to equation 1. The theoretical lines generated are shown in Fig. 1 and the values obtained for k_2 and K_1 are shown in Table 1. The values for the constants determined through the end point method (equation 3) did not differ significantly from those calculated above (Table 1).

Chemical modification of α-chymotrypsin by DIMBOA

Inactivation of α-chymotrypsin by DIMBOA was carried out in the presence of different concentrations of N-acetyl-L-phenylalanine ethyl ester (AFEE), a synthetic substrate of the enzyme. The results (Table 2) showed that AFEE protected the enzyme against DIMBOA inactivation, suggesting that the reaction occurred at or near the active site. Aminoacid analysis of α-

chymotrypsin inactivated by DIMBOA, as compared with native α-chymotrypsin, indicated that only the number of lysine residues was significantly reduced by reaction with DIMBOA (Table 3).

Tryptophan and methionine were not detected by this method. It is however, unlikely that these aminoacids suffer changes since they do not react with DIMBOA in aqueous solutions [10, 11]. Lysine, on the other hand, reacts with DIMBOA in aqueous solution with the intermediacy of the open chain aldol 2 [12] and lysine residues in α-chymotrypsin are located in the outer surface of the enzyme [13]. Although normal serine residues in enzymes are not expected to react with DIMBOA [11], the enhanced nucleophilicity of the active site serine in α-chymotrypsin [14] warranted the parallel determination of active site serine titre [15] and enzyme activity in the presence of excess DIMBOA. The kinetic patterns for both processes were similar (Fig. 2), suggesting that the inactivation of α-chymotrypsin by DIMBOA was due to its reaction with the active site serine. The precission of aminoacid analysis did not allow the detection of the modification of this single serine residue.

DIMBOA reacts with nucleophiles either through its hydroxamic nitrogen or through the reactive aldehyde group of aldol 2, in equilibrium with it in solution [16]. Hence, inactivation experiments were carried out with 4-hydroxy-2,7-dimethoxy-1,4-benzoxazinone 3, a compound that canno form aldol 2, and hence does not generate a reactive aldehyde group. Compound 3 did not inactive α-chymotrypsin in the range of concentrations studied (5 to 20 mM), suggesting that the reaction

of DIMBOA with α-chymotrypsin occurred through the aldol tautomer 2. In support of this conclusion, α-chymotrypsin was inactivated by phenylglyoxal (data not shown), a compound containing an α,ß-dicarbonyl function similar to that of aldol 2.

Insect digestive enzymes have been suggested as possible target sites for the action of plant defence chemicals [17]. Its appears likely that the toxicity of DIMBOA towards phytophagous insects such as O. nubilalis is related to its capacity to inhibit serine or thiol [18] proteinases in the larval digestive tract.

EXPERIMENTAL

Enzymes and reagents

α-Chymotrypsin (3 x crystallized), N-acetyl-L-phenylalanine-ethyl ester (AFEE), N-carbobenzyl-L-tyrosine-p-nitrophenyl ester, N-trans-cinnamoylimidazol, MOPS and $CaCl_2$ were purchased from Sigma. 4-Hydroxy-2,7-dimethoxy-1,4-benzoxazin-3-one was a gift from Dr. J. Atkinson from the University of Ottawa, Canada.

2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA)

This compound was isolated from ethereal extracts of *Zea mays* L. cv. T 129s, as described [19].

Assay of α-chymotrypsin activity

The hydrolytic activity of α-chymotrypsin was measured at 30° with N-carbobenzyl-L-tyrosine-p-nitrophenyl ester as substrate. The increase in absorbance at 400 nm due to the release of p-nitrophenol was followed [20]. The reaction mixture consisted of 14 µl of 3.2×10^{-4} M substrate, 2.6 ml of 0.1 M MOPS buffer with 5 mM $CaCl_2$ pH 7.5 (buffer A), 350 µl of CH_3CN and 5 µl of 3.2×10^{-6} M α-chymotrypsin. The non-enzymatic hydrolysis of the substrate was taken into account by using substrate in the reference cuvette.

Inactivation of α-chymotrypsin by DIMBOA

The reactions were followed under pseudo-first order conditions with an excess DIMBOA, by taking 5 µl from the

reaction mixture (1 ml) after appropriate time intervals and measuring the decrease in the enzymatic activity by the procedure previously described.

Substrate protection

A solution (14 µl) of 3.2 x 10^{-6} M α-chymotrypsin in 1 ml buffer A was incubated at 30° in the presence of 30 mM DIMBOA and concentrations of AFEE ranging from 0 to 40 mM. Aliquots (5 µl) were withdrawn from the reaction mixture and activity was measured according to the assay previously described.

Amino acid analysis

A mixture consisting of 1.6 x 10^{-4} M α-chymotrypsin and 40 mM DIMBOA was incubated in buffer A at 30° for 12 h. After this time, α-chymotrypsin had lost its activity. The reaction mixture was filtered through a Sephadex G-25 column equilibrated with the same buffer, in order to eliminate excess DIMBOA. The filtrate was concentrated by ultrafiltration and then lyophilised. The protein was hydrolysed with 6 M HCl at 110° for 24 h and analysed in a Beckman amino acid analyzer model 120 C. A sample of native α-chymotrypsin was submitted to the same procedure.

Titration of active site serine of α-chymotrypsin

The titre of active site serine of α-chymotrypsin was determined as described [15]. To a mixture containing 2.9 ml of 0.1 M acetate buffer with 3.2 % of CH_3CN pH 5 and 24 µl of 10 mM N-trans-cinnamoylimidazol (ϵ = 9,115 $M^{-1}cm^{-1}$), different volumes

of 3.2×10^{-4} M of α-chymotrypsin were added. Decrease in absorbance at 325 nm was measured with respect to a control without enzyme. The enzyme concentration was measured at 280 nm ($\epsilon = 50,000\ M^{-1}cm^{-1}$) [21]. A relation of 0.97 mole of serine per mol α-chymotrypsin was obtained for native α-chymotrypsin.

Kinetics of the loss of active site serine titre of α-chymotrypsin in the presence of DIMBOA

After different time intervals 200 µl aliquots were withdrawn from a reaction mixture consisting of 0.5 ml of 3.2×10^{-4} M α-chymotrypsin, 1.9 ml of buffer A and 100 µl of 0.5 M DIMBOA in DMSO. The aliquots were filtered through a Sephadex G-25 column as described [22] in order to eliminate excess DIMBOA. Active site serine titre and enzyme activity were measured in the filtrates after different time intervals according to the assays previously described.

Acknowledgements We gratefully acknowledge the financial support of International Program in the Chemical Sciences (Uppsala University), Agency for International Development and FONDECYT.

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Legends to figures:

Fig. 1. Kinetics of α-chymotrypsin inactivation by DIMBOA at different concentrations: 0 (□); 10 (○); 15 (●); 20 (△) and 40 (▲) mM. The curves represent the least-squares fit of the experimental points to equation 1. Initial specific activity was 4.9 µmol/min mg protein.

Fig. 2. Proportionality of α-chymotrypsin activity and serine titre as percentage of initial values, during the inactivation of the enzyme by DIMBOA. Initial specific activity was 4.9 µmol/min mg protein and initial serine titre was 0.97 mol serine/mol enzyme.

Table 1. k_2 and K_i values from inactivation kinetics of α-chymotrypsin by DIMBOA in 0.1 M MOPS buffer with 5 mM $CaCl_2$, pH 7.5 at 30° C.*

DIMBOA (mM)	$^{+}k_2$	$^{+}Ki \times 10^{-3}$
10	0.106 ± 0.024	6.4 ± 2.5
15	0.102 ± 0.024	5.3 ± 3.1
20	0.116 ± 0.016	7.8 ± 2.0
40	0.093 ± 0.03	6.0 ± 2.0

* $k_d = 8 \times 10^{-2}$ min^{-1} (ref. 4).

\+ k_2 and Ki values determined through the end point method (equation 3) were 0.076 ± 0.0063 min^{-1} and 5.2 ± 1.3 x 10^{-2} M, respectively.

Table 2. Relative activities of α-chymotrypsin inactivated by 30 mM DIMBOA after 3 h of incubation at 30° pH 7.5 in the presence of different concentrations of N-acetyl-L-phenylalanine ethyl ester (AFEE).

AFEE (mM)	Relative activity (%)*
0	18
10	37
20	51.5
40	63

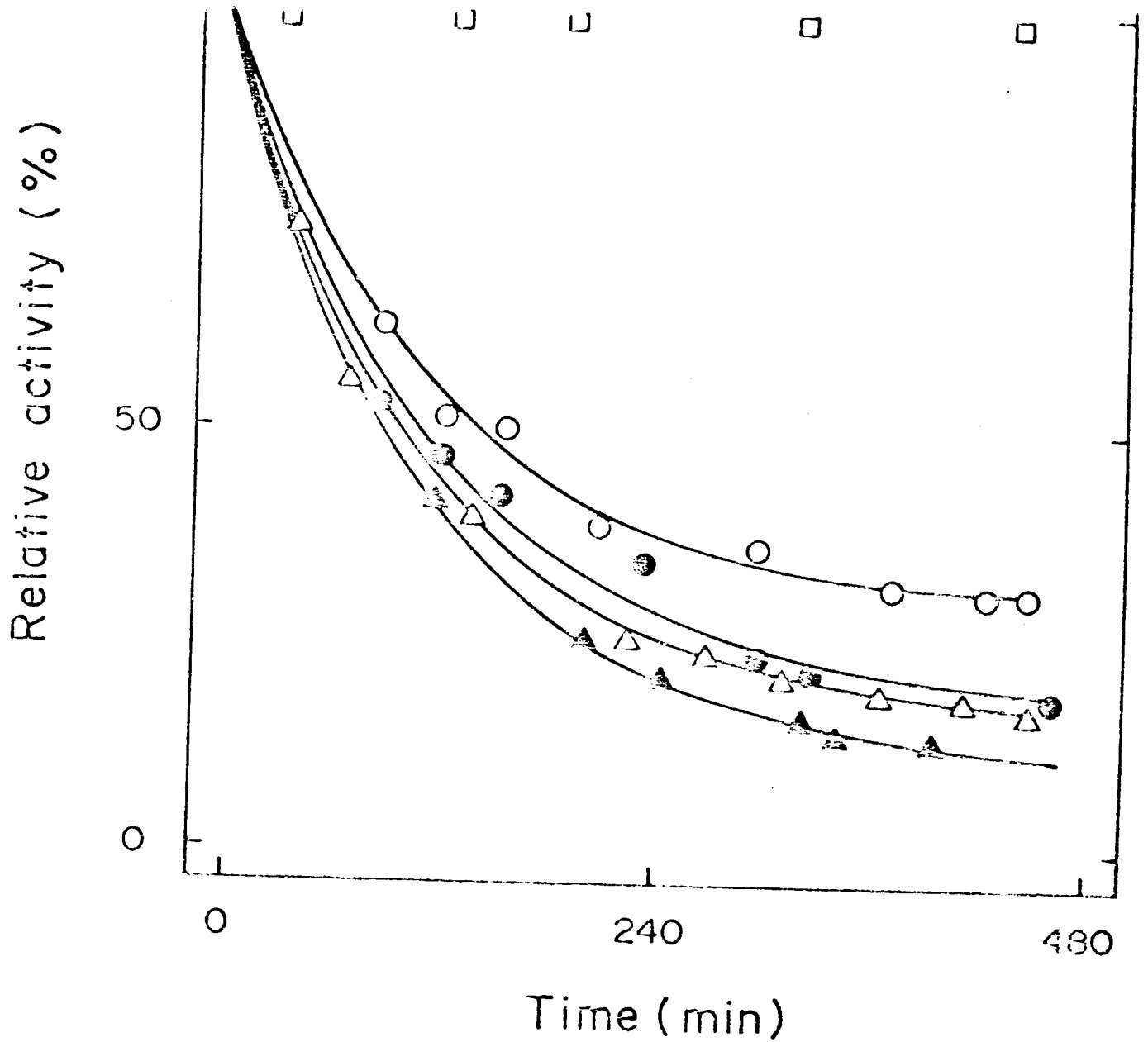
*Initial specific activity was 4.9 µmol/min mg protein.

Table 3. Amino acid analysis of native α-chymotrypsin and α-chymotrypsin modified by DIMBOA.

Aminoacid	Native* α-chymotrypsin	Modified* α-chymotrypsin
Trp+	-	-
Lys	12.9 ± 0.1	4.9 ± 0.6
Arg	3.0	3.0
Met+	-	-
Asp	21.4 ± 0.2	22.3 ± 0.2
Thr	22.0	22.0
Ser	27.5 ± 2.1	25.2 ± 1.1
Glu	18.4 ± 0.6	17.8 ± 0.2
Pro	9.6 ± 0.1	9.2 ± 0.1
Gly	29.0 ± 0.9	27.7 ± 0.6
Ala	24.8 ± 0.1	25.9 ± 0.5
Val	20.6 ± 0.1	21.1 ± 0.2
Ile	6.6 ± 0.1	7.2 ± 0.1
Leu	17.0 ± 0.1	16.7 ± 0.1
Tyr	2.8 ± 0.1	2.8 ± 0.2
Phe	4.9 ± 0.1	5.0 ± 0.1

* Residues per mole protein. Data was normalized with respect to arginine and threonine.

+ Not detected by this method.



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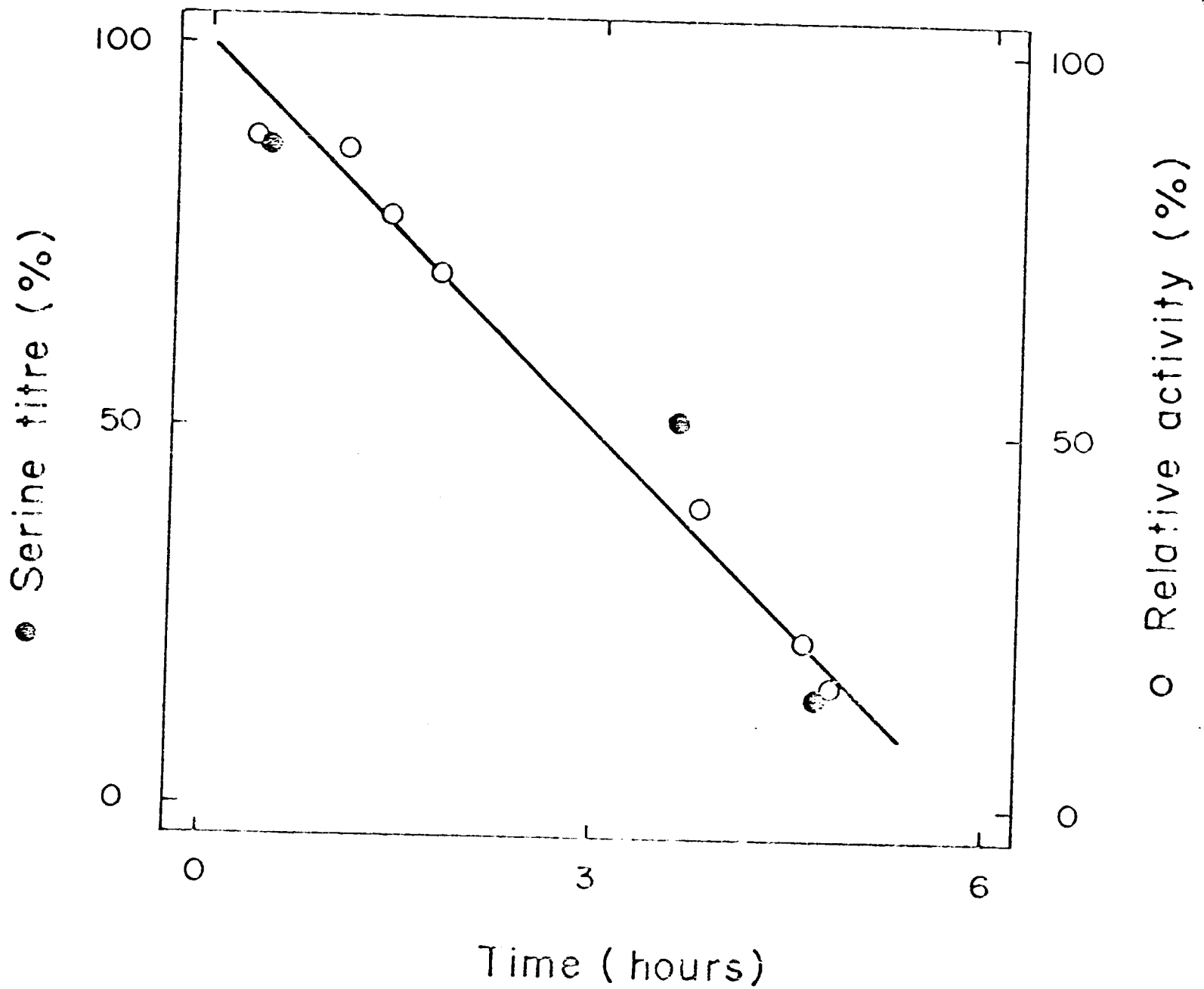
100
50
0
● Serine titre (%)
100
50
0
○ Relative activity (%)
0
3
6
Time (hours)

ALLELOPATHIC EFFECT OF HYDROXAMIC ACIDS FROM CEREALS ON OAT *A. SATIVA* L. AND WILD OAT *AVENA FATUA* L.

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Key Word Index: Allelopathy, hydroxamic acids, benzoxazinones, wheat, *Avena fatua* L., *Avena sativa* L.

Abstract. Wild oat *Avena fatua* L. is a worldwide weed associated with wheat. 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA, 1), the main hydroxamic acid isolated from wheat, and its decomposition product 6-methoxy-benzoxazolin-2-one (MBOA, 2), inhibited 50% root growth of *A. fatua* at concentrations of 0.7 and 0.5 mM respectively. MBOA also inhibited seed germination of *A. fatua* at all concentration tested. It stimulated root growth in *A. sativa* at concentrations below about 1.5 mM and inhibited it at higher concentration. Pulse experiments with DIMBOA indicated that it decomposed to MBOA in *A. fatua* seeds within a period of 48 h. Uptake by *A. fatua* seeds of MBOA, DIMBOA and water showed similar kinetic patterns. However MBOA was taken up preferentially to DIMBOA. MBOA uptake depends on the afterripening time of the seed.

The potential exploitation of hydroxamic acids from wheat as allelochemicals in the control of *A. fatua* is discussed.

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INTRODUCTION

Wild oat, Avena fatua L., is one of the most troublesome annual grass weeds of wheat and other cereals [1]. Infestation by wild oat causes extensive cereal-crop yield losses [2-4]. Numerous pre and postemergence herbicides have been developed for the control of wild oat in various crops [5,6]. An alternative way to control weeds in crops is through allelochemicals that inhibit the germination and growth of weed species, and are produced and released by growing crop-plants or their residues. This alternative for weed control is receiving increased attention due to its potential in avoiding adverse environmental effects [7-9].

Hydroxamic acids (Hx) isolated from wheat and other cereals, have been involved in plant resistance to pests and diseases [10]. Recently, the Hx isolated from rye (Secale cereale L.) 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA, 3), and its decomposition product benzoxazolin-2-one (BOA, 4), were associated with the allelopathic effects exhibited by this crop [11,12].

This paper describes the phytotoxic effect of DIMBOA (1), the main Hx in wheat, and its decomposition product MBOA (2), on A. fatua, a worldwide weed affecting wheat, and on A.sativa a crop normally rotated with it.

RESULTS

Effect on root length

DIMBOA inhibited root growth of *A. fatua* (I_{50} = 0.7 mM). The inhibitory effect was significantly smaller in *A. sativa*. MBOA also inhibited the root growth of *A. fatua* (I_{50} = 0.5 mM). However, it stimulated root growth of *A. sativa* at concentrations below about 1.5 mM, and inhibited it above this concentration (Fig. 1).

DIMBOA decomposes in buffered water to MBOA with a half life of *ca*. 27 h at pH 5 [13]. Since bioassays were carried out in distilled water pH 5, the effects exhibited by DIMBOA may be questioned. Quantitation of DIMBOA and MBOA in *A. fatua* seeds was carried out. *A. fatua* seeds were submitted to a pulse of DIMBOA (4 mM, 4 h) and then transfered to a medium containing only water. HPLC analysis of DIMBOA and MBOA was performed in the seeds after different intervals of imbibition time (Table 1). The results indicated that most DIMBOA taken up by the seeds decomposed to MBOA within 48 h.

Effect on seed germination

Germination of *A. fatua* was not affected by DIMBOA up to a concentration of 2 mM, after which inhibition reached completeness at 8 mM. In contradistinction, all concentrations of MBOA inhibited germination with completeness being reached at 4 mM (Fig. 2).

Kinetics of uptake of DIMBOA, MBOA and water by *A. fatua* seeds.

Kinetics of DIMBOA and MBOA uptake by *A. fatua* seeds were performed in order to establish whether differences existed in the absorption of the compounds. The experiments were carried out with *A. fatua* seeds that had not completed their afterripening period and hence were in a dormant state.

Uptake of water and test compounds followed similar patterns. However, uptake of MBOA was significantly faster and more extensive than that of DIMBOA (Fig. 3).

Uptake of MBOA by dormant and nondormant *A. fatua* seeds was also measured (Fig. 4). Significant differences were found.

DISCUSSION

Release and uptake of Hx

One of the basic criteria for a phytotoxic compound to be involved in allelopathic effects is that it must be released from the producing plant to the soil and absorbed by the target seed or plant [14]. The presence of allelochemicals in the soil is due mainly to root exudation by the producing plant or release from plant residues. Information is not available on the liberation of hydroxamic acids (Hx) to the soil by root exudation. However, there are reports involving rye residues and wheat straw in allelopathy [15, 16]. Moreover, it was recently reported that DIBOA, the main Hx in rye extracts and its decomposition product BOA, are phytotoxic towards several mono and dicotiledoneous plants [11].

The decomposition of DIMBOA inside the seed (Table 1) as well as the preferential uptake of MBOA over DIMBOA (Fig. 4) suggest that if Hx are released to the soil, the compounds most likely to act as allelochemicals are the corresponding benzoxazolinones.

Significant differences in MBOA uptake were found between dormant and nondormant A. fatua seeds (Fig. 4). For example, after 48 h of imbibition in 0.5 mM MBOA (corresponding to I_{50}) 0.23 µg of MBOA were found inside each nondormant seed. In dormant seeds, this quantity of MBOA would be expected to be reached with an MBOA concentration in the imbibition medium 6-fold lower (Fig. 4). These results indicate that the

concentration of MBOA necessary to produce phytotoxic effects on A. fatua depends on the length of the afterripening period to which the seed being tested has been subjected. The change in MBOA uptake with the length of the afterripening period of A. fatua seeds, may be related to changes in the fatty acid composition of membranes, and hence with changes in their permeability [17].

Mechanism of phytotoxicity of Hx

Although Hx are toxic towards a wide range of organism [10], the mechanisms of their toxicity are not yeat known. Two alternative have been reported that may account for phytotoxic effects. DIMBOA and MBOA modified the binding affinity of 1-naphtyl acetic acid, a synthetic auxin analogue, to membrane preparations of corn coleoptile, the modification being correlated with the inhibition by Hx of the auxin-induced growth in oat coleoptile sections [18]. On the other hand, DIMBOA inhibited both cyclic and noncyclic photophosphorylation in spinach chloroplasts [19]. Both bioassays employed in this work, seed germination and root growth, were performed in the dark. Thus, both processes are dependent solely on seed reserves with no significant participation of photosynthesis. Hence, it seems more likely that phytotoxicity of Hx is related with their intereference on the normal activity of auxin. Further studies are underway to test this hypothesis.

Potential uses of Hx as allelochemicals

Strategies for utilizing allelopathy as an aid in crop production are receiving greater attention [7-9]. Within these, weed control and crop stimulation have aroused greatest interest. In this paper, it is shown that Hx isolated from wheat inhibited root growth and seed germination of *A. fatua*, an annual grass weed asociated to this crop. It was also shown that MBOA at low concentrations stimulated root growth of *A. sativa*, a crop normally rotated with wheat. These results suggest the potential beneficial involvement of Hx as weed inhibitors through root exudation and as crop-plant stimulators through wheat residues. Further work on the release, dispersal and uptake of Hx should be undertaken before definitive proposals are made.

EXPERIMENTAL

Compounds

DIMBOA was isolated as described [19] from Et_2O extracts of 7 day-old seedlings of T. durum cv. SNA-3, grown in a greenhouse at 25 ± 3°. MBOA was synthesized as described [20].

Seeds

Seeds of wild oat A. fatua were obtained from the Agricultural Experimental Station Quilamapu (INIA), and were collected in 1986 and stored under dry conditions for two years. These seeds were nondormant and had a germination rate higher than 95%. Dormant A. fatua seeds that had not completed their afterripening period, were obtained as the F_1 from the seeds collected in 1986. The rates of germination in this case was lower than 5%. Seeds of A. sativa were obtained from the local market.

Germination

Dehulled seeds of A. fatua were treated with 2% NaClO and washed with excess H_2O. The seeds were placed in 10 cm Petri dishes (10 seeds per dish) over a disk of Whatman N°1 filter paper, and 2.5 ml H_2O were added. The dishes were incubated in the darkness at 20 ± 30°. Seeds were considered germinated when the radicle appeared through the testa.

Bioassays

Bioassays were carried out in Petri dishes containing one disk of Whatman N°1 filter paper. Appropriate solutions of DIMBOA and MBOA in acetone were added to the filter paper. Pure acetone, similarly applied to the filter paper was used as control. After solvent evaporation 2.5 ml of distilled water were added making the solution of the proper concentration in test compound, and 10 seeds of *A. fatua* or *A. sativa* were placed in each disk. Root length and seed germination were determined after 3 and 6 days of incubation in the dark at 20 ± 3°, respectively. Each bioassay was carried out in triplicates and the t-test was applied for significant differences.

Kinetics of uptake of DIMBOA, MBOA and water by *A. fatua* seeds

Ten dehulled dry seeds of dormant *A. fatua* were weighed and placed for imbibition in a 4 mM solution of test compound as described in the bioassay. After different time intervals, the seeds were washed twice with water, dried with paper towel and weighed again. Water uptake by the seeds was determined from the difference in weight. Seeds were homogenized with mortar and pestle in 2 ml MeOH. The extracts were centrifuged at 14,000 g in a microfuge for 15 min, filtered through a millipore filter tye LSWP (5 µm pore diameter) and then through a Sep-pack C18 cartridge, in order to eliminate large particles and hydrophobic compounds that could interfere in the HPLC analysis. After this treatment, the methanolic extract was evaporated to dryness and redissolved in 100 µl pure MeOH for injection.

HPLC analysis of DIMBOA and MBOA

HPLC analysis was performed either in a Varian 5000 liquid chromatograph equipped with a C18 Micro Pak MCH-10 column (300 x 4 mm) and uv detector (fixed at 254 nm) or in a Shimadzu LC-6A liquid chromatograph, equipped with a Lichrocart C18 column (125 x 4 mm) and a variable uv-vis detector (fixed at 290 nm). The mobile phase consisted of a mixture of 70% acidic water (5 mM H_3PO_4, pH 2.5) and 30% MeOH. Flow rate was 1.2 ml/min. Retention times for DIMBOA and MBOA were 6.0 and 10.8 min respectively. The volume injected varied between 10 and 100 µl depending on the sample. Concentration of DIMBOA and MBOA in the samples were obtained by extrapolation from the corresponding calibration curves. All samples were analyzed by triplicate.

Decomposition of DIMBOA in *A. fatua* seeds

Ten dehulled seeds of *A. fatua* were placed for imbibition in a 4 mM solution of DIMBOA for 4 h. After this time, the seeds were washed twice with water, dried with paper towel and placed in Petri dishes containing only water. After different time intervals HPLC analysis of DIMBOA and MBOA were performed as described previously.

Acknowledgements. The technical assistance of Ms. Patricia Muñoz and the financial support from the International Program in the Chemical Sciences-Uppsala University and the Agency for Internacional Development is gratefully acknowledged.

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LEGENDS TO FIGURES

Fig. 1. Inhibition and stimulation of root growth of wild oat *A. fatua* (●) and oat *A. sativa* (O) by MBOA (——) and DIMBOA (---) after 3 days incubation in the dark at 20 + 3°. LSD relative to control for *A. fatua*: DIMBOA (5-5%) and MBOA (5-9%). LSD relative to control for *A. sativa*: DIMBOA (5-24%) and MBOA (5-17%).

Fig. 2. Inhibition of germination of *A. fatua* by MBOA (●) and by DIMBOA (O) after 6 days incubation in the dark at 20 + 3°.

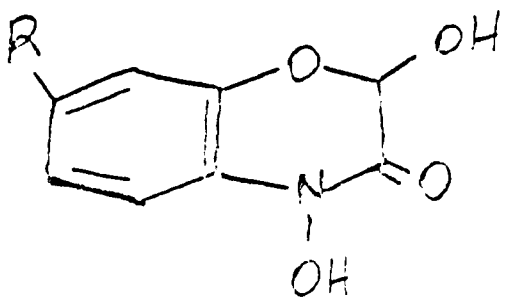
Fig. 3. Kinetics of uptake of DIMBOA (O), MBOA (□) and water (●) by dormant *A. fatua* seeds. The concentration of test compounds was 4 mM.

Fig. 4. Uptake of MBOA by dormant (O) and nondormant (●) *A. fatua* seeds.

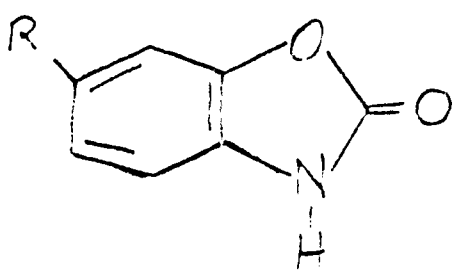
Table 1. Decomposition of DIMBOA in *A.fatua* seeds

Incubation with 4 mM DIMBOA (h)	Inbibition in water (h)	µg DIMBOA/ g dry seed	µg MBOA/ g dry seed
4	0	11.3 ± 2.0	6.3 ± 0.5
4	24	6.2 ± 0.3	11.6 ± 1.5
4	48	1.1 ± 0.1	16.1 ± 2

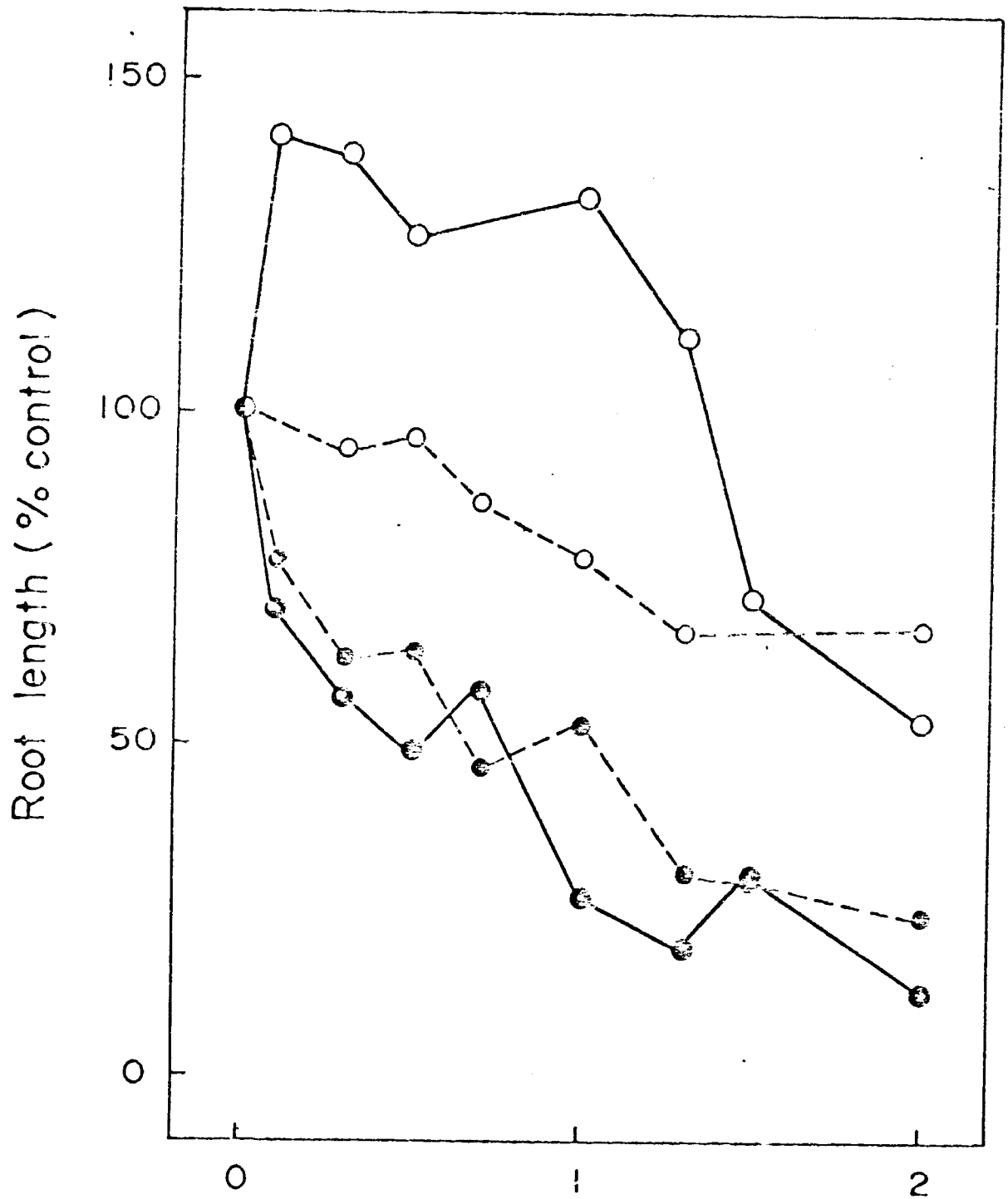
Structures

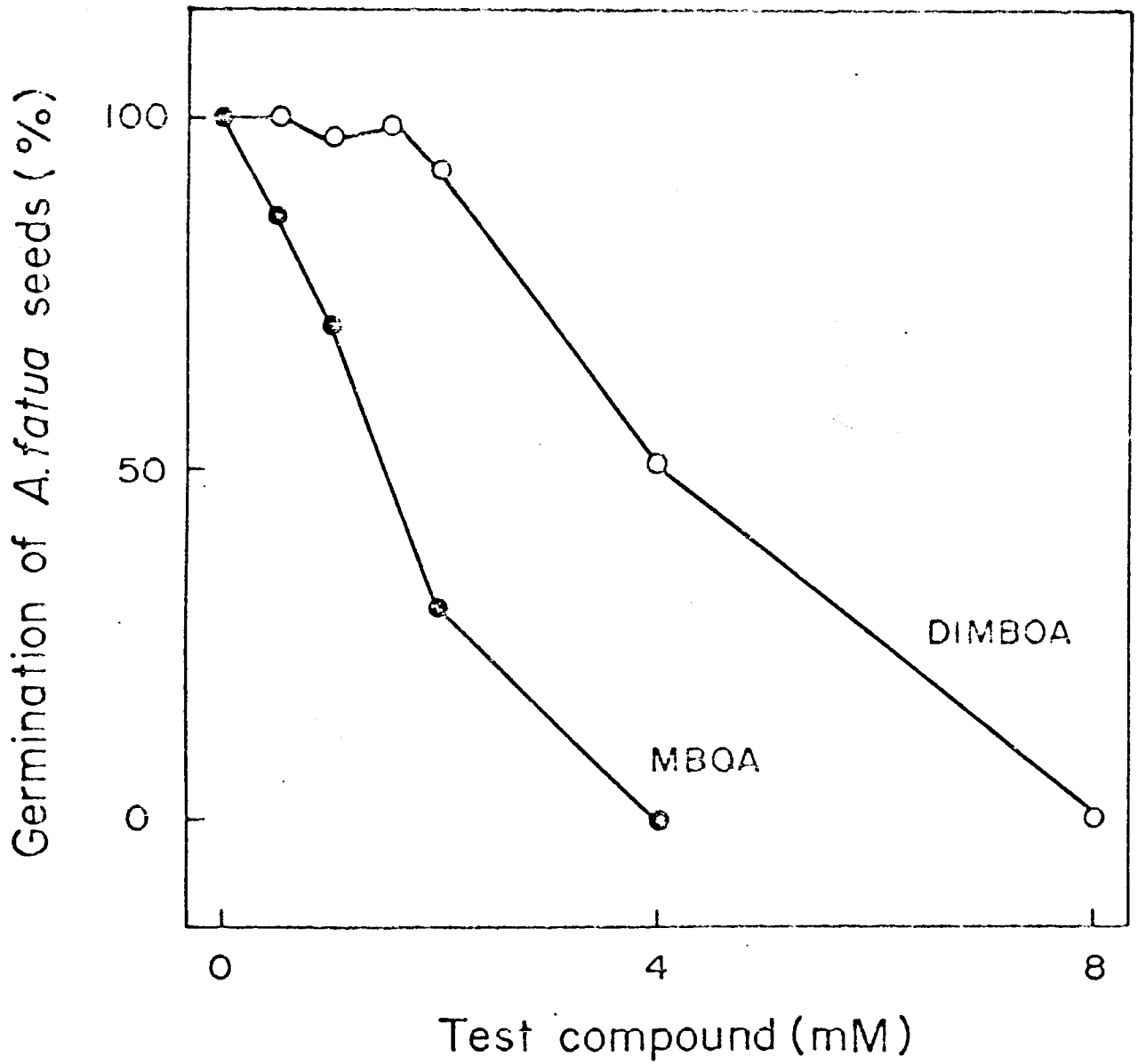


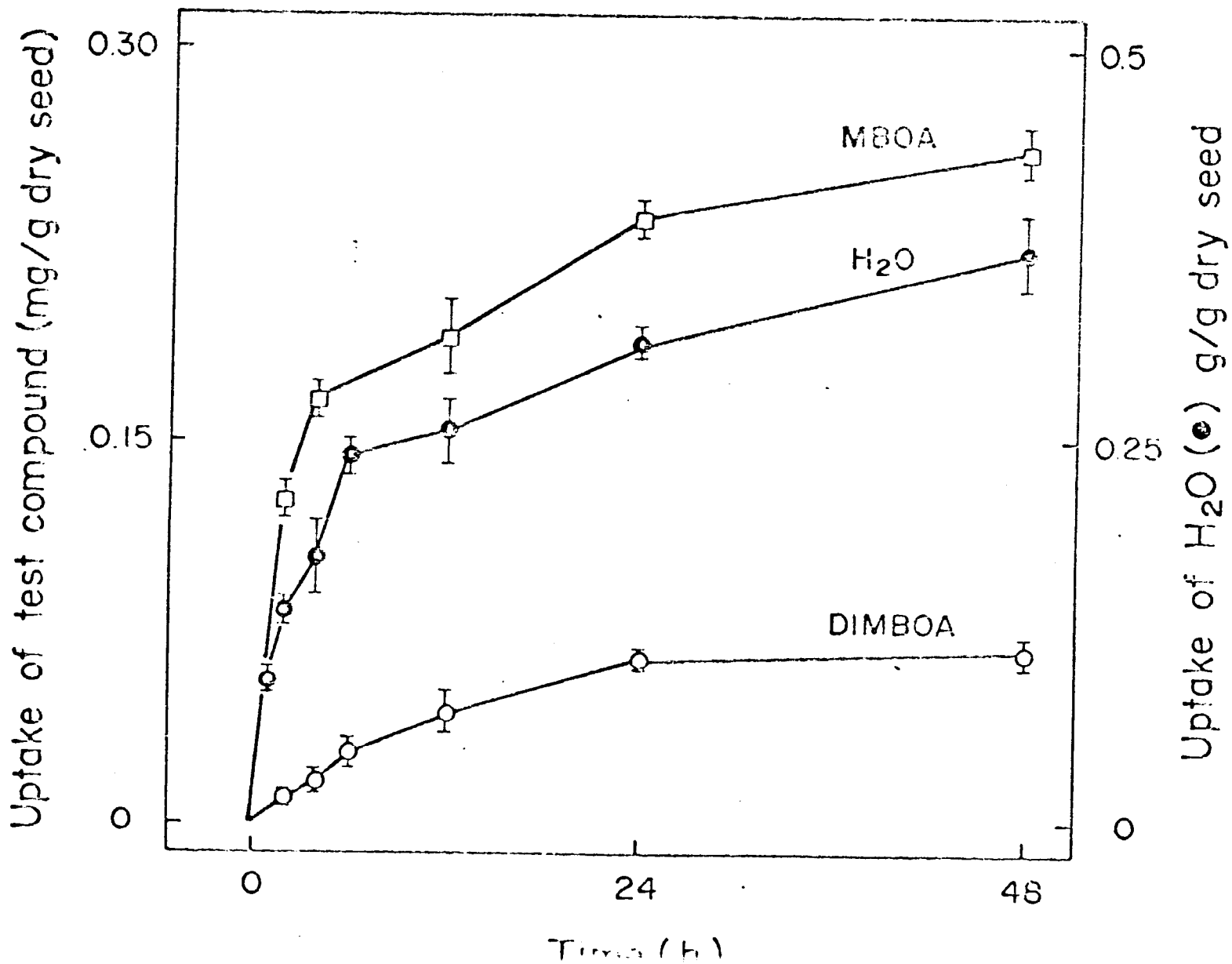
	R
1	OCH_3
3	H



	R
2	OCH_3
4	H


Root length (% control)
150
100
50
0
0
1
2


Germination of A. fatua seeds (%)
100
50
0
0
4
8
Test compound (mM)
DIMBOA
MBOA


Uptake of test compound (mg/g dry seed)
0.30
0.15
0
MBOA
H_2O
DIMBOA
0.5
0.25
0
Uptake of H_2O (●) g/g dry seed
0
24
48

7.404

Research Report No. 1

Grant: DPE-5542-G-SS-8077-00

Period covered:

From: August 30, 1988

To: February 28, 1989

Preliminary remarks

The research proposal that lead to this grant was submitted in August 1986. Part of the research proposed was carried out by us between the submittal date and the date of commencement of the grant (August 1988). The results obtained will be briefly described, as a background to research carried out under the grant.

Delays in getting the grant started are reflected in a few weeks' delay in the submittal of this report.

Wheat-aphid-virus interactions mediated by hydroxamic acids (Hx)

Background

Virus transmission by aphids to oats was found to be ineffective if the aphid fed from the plant phloem for a period shorter than a certain threshold [Scheller & Shukle (1986) Entomol. Exp. Appl. 40: 189-195]. Based on these results, we postulated that hydroxamic acids (Hx) could be important in preventing viral infection since they had been shown to be antifeedants towards aphids in artificial diets [Argandoña, Corcuera, Niemeyer & Campbell (1983) Entomol. Exp. Appl. 34: 134-138].

Results 1986-1988

In experiments carried out in conjunction with scientists in Hamburg, an antifeedant effect of Hx was also demonstrated with aphids feeding on plants differing in Hx levels [Niemeyer, Pesel, Franke & Francke (1989) Phytochemistry, in press].

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Results under the AID grant

We have undertaken, in collaboration with agronomists at INIA-Chile (Instituto de Investigaciones Agropecuarias), a survey of bread wheats in relation to their Hx levels and their tolerance to barley yellow dwarf virus. This latter parameter was determined by artificially infesting with viruliferous aphids wheat plants in Feekes growth stage 7, and weighing an hectoliter of grain produced. The preliminary data is encouraging. Figure 1 shows that, with the exception of lines 4 and 7, susceptible lines show low Hx levels and resistant lines high Hx levels. It is likely that in lines 4 and 7 factors other than Hx play an important role in BYDV tolerance.

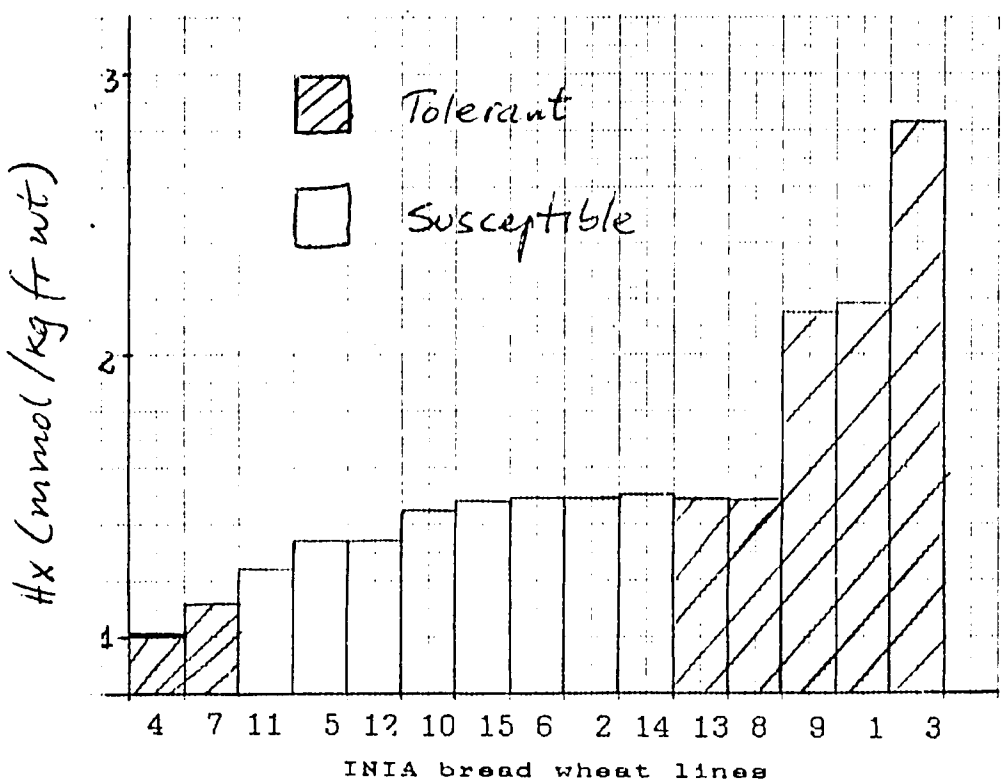

Fig. 1. Tolerance to BYDV and Hx levels in INIA bread wheat lines

Choice experiments were carried out in which young aphid adults inside a clip cage were allowed to choose between leaves of wheat cultivars differing in Hx levels. Preliminary results show a tendency for plants with lower Hx levels to become more infested by aphids (Table 1).

Table 1

Pairs in choice experiment

Line	Anza	Millaleu	Anza	Mexifen	Mexifen	Millaleu
Hx level +	2.8	0.7	2.8	1.4	1.4	0.7
Reaction to BYDV *	T	S	T	I	I	S
% of aphids settled	35	65	38	62	52	48

+ mmol/kg fr. wt.
* T= tolerant, S= susceptible, I= intermediate.

Future experiments

The range of lines and cultivars used in the Hx level-BYDV tolerance survey will be expanded. Additional choice experiments will be carried out and the results will be related to BYDV infection levels.

Toxicity and reactivity of hydroxamic acids

Background

We postulated that toxicity of Hx is related to the inhibition of enzymes in key metabolic processes in the recipient organism, through their rection with nucleophilic sites in the enzymes [Niemeyer, Corcuera & Pérez (1982) Phytochemistry 21: 2287-2289]. This hypothesis has received independent support. Thus, it was demonstrated that DIMBOA, the main Hx in wheat and maize, inhibits proteinases isolated from the gut of the European corn borer [Campos, Houseman, Atkinson & Arnason (1988) personal communication].

The reaction of DIMBOA with thiols, nucleophiles modelling sulfhydryl groups in enzymes, was studied in this context [Pérez & Niemeyer (1985) Phytochemistry 24: 2963-2966].

Results 1986-1988

We also studied the reactivity of DIMBOA with amines, as nucleophiles modelling lysine residues of enzymes. DIMBOA reacted with primary amines to give addition compounds with two imino groups. Kinetic studies with DIMBOA and with the DIMBOA analogues 2,7-dimethoxy-1,4-benzoxazin-3-one and 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one indicated that the reaction occurred with intermediacy of the aldol tautomer of DIMBOA. This reactivity could be related to the anti-inflammatory effects of DIMBOA and related molecules [Pérez & Niemeyer (1989) Phytochemistry, in press].

Results under the AID grant

The inactivation of papain, a thiol protease, by DIMBOA, was studied. The process involved the reaction of DIMBOA with a non-essential serine residue followed by its reaction with the essential Cys-25. The nitrogen atom in DIMBOA was the active electrophile [Pérez & Niemeyer (1989) Phytochemistry, in press, galley proofs included].

Future experiments

Serine is an essential residue of α-chymotrypsin, a widely distributed digestive enzyme. The effect of DIMBOA on this enzyme will be studied. The results will be relevant to the interpretation of the toxicity of DIMBOA based on the disruption of digestive processes in phytophagous insects.

Hydroxamic acid levels in wheat and its relatives

Background

Breeding for high levels of Hx in wheat depends on the availability of suitable parental genotypes.

A partial screening for Hx levels of the genus Triticum was carried out using a colorimetric method [Niemeyer (1988) Euphytica 37: 289-293]. Some primitive diploid species were found with promising Hx levels.

Results 1986-1988

A method for quantifying hudroxamic acids utilizing high performance liquid chromatography was developed in collaboration with scientists in Hamburg [Niemeyer, Pesel, Copaja, Bravo, Franke & Francke (1989) Phytochemistry, in press].

This method was used in the screening of wheat cultivars currently sown in Chile for Hx levels, persistance of Hx along the development of the plant, ratio of DIMBOA to DIBOA (7-demethoxylated analogue of DIMBOA), and inducibility of Hx by aphid feeding. This later property was discovered in joint work with colleagues in Hamburg [Niemeyer, Pesel, Copaja, Bravo, Franke & Francke (1989) Phytochemistry, in press].

Results under the AID grant

Fifty four wheat cultivars were screened. Hx levels go through a maximum when the seedling is between 4 and 7 days old. At the maximum, the levels found in the cultivars examined ranged from 4 (Talafen) to 35 (Lanco) mmol/kg fr. wt.

When cultivars were examined for persistance of DIMBOA with seedling age, the decrease factor from the 4th to the 8th day ranged from 20 (Chapelle Desprez) to 1 (Canelo). The results are encouraging since they point to the possible existance of plants with high Hx levels at the stage when they are attacked by aphids in the field.

The most abundant Hx in wheat are DIMBOA and DIBOA, the former being the most toxic. Hence, a plant is desired in which the ratio DIMBOA/DIBOA is maximized. In 4-day old seedlings of the cultivars screened, this ratio ranged from 13 (Lilifen) to 95 (Millafen).

Inducibility by aphid feeding was also assessed, the ratio of DIMBOA in an infested vs. a non-infested 7-day old seedling ranging from 0.6 (Malifen) to 4 (Cisne).

Future experiments

The screening outlined will be continued. Hx levels will be followed until later stages of development of the plant, and the inducibility of Hx by aphids will be further studied.

A wider range of *Triticum* material will be screened, and perennial Triticeae will also be examined.

Date 4.2.1989

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REACTION OF DIMBOA, A RESISTANCE FACTOR FROM CEREALS, WITH PAPAIN

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Key Word Index—Gramineae; resistance factor; hydroxamic acids; 1,4-benzoxazin-3-ones; thiols; papain inactivation; DIMBOA.

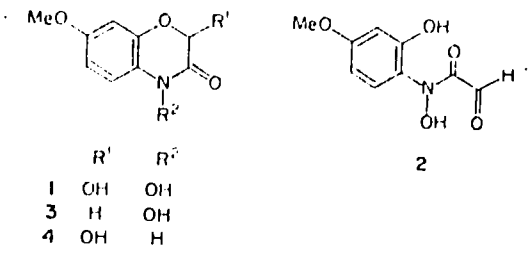
Abstract—2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), a hydroxamic acid from cereals involved in host plant resistance to insects, inactivated papain (EC 3.4.2.2). Semilog plots of the residual activity of papain in the presence of an excess of DIMBOA as a function of time were concave downwards. Cysteine and serine were significantly modified by DIMBOA inactivation. Loss of enzyme activity by reaction with DIMBOA parallelled loss of thiol titre of the enzyme. DTT partially restored the activity of papain inactivated by DIMBOA. A kinetic model involving the reaction of a non-essential serine residue of papain followed by the reaction of essential Cys-25 with DIMBOA quantitatively accounted for these results. 4-Hydroxy-7-methoxy-1,4-benzoxazin-3-one inactivated papain in a manner similar to DIMBOA, whereas 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one showed no effect on papain. These results suggest that the inactivation of papain by DIMBOA occurs by interaction of the sulphur atom of Cys-25 with the hydroxamic nitrogen of DIMBOA. The results are discussed in relation to the toxicity of DIMBOA towards insects.

INTRODUCTION

Hydroxamic acids (Hx) such as 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA, 1), play a defensive role in wheat and maize plants against insects [1–3]. The toxicity of these acids is associated with their interference with key metabolic processes, such as energy transduction in mitochondria [4].

The chemical properties of DIMBOA in solution [5, 6] suggested that enzymic inhibitions may be due to the reaction of DIMBOA with nucleophilic residues in the enzymes [7]. The reaction of DIMBOA with thiols in aqueous solutions was described and a mechanism for it proposed [8, 9]. It remains to be demonstrated whether enzymic sulphydryl groups are able to react with DIMBOA.

An ideal model system for the study of the reaction of DIMBOA with a sulphydryl group in an enzyme is papain (EC 3.4.2.2), since it has a single free cysteine residue, that of the active site (Cys-25), and its structure and mechanism of catalysis are well known [10, 11]. In this work we show that DIMBOA inactivates papain through reaction of its nitrogen atom with Cys-25.



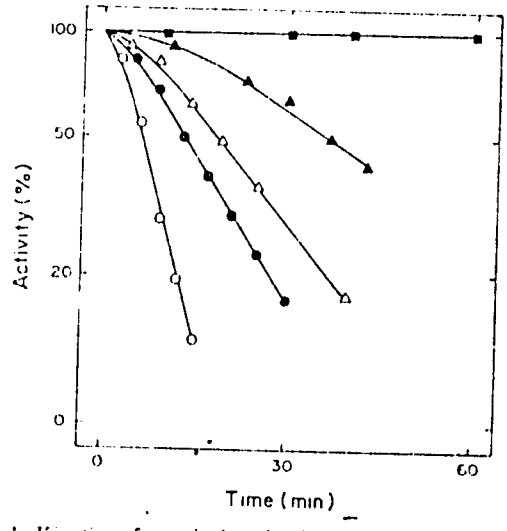


Fig. 1. Kinetics of papain inactivation at different DIMBOA concentrations: 0 (■), 3 (▲), 5 (△), 8 (●), and 15 mM (○), in 50 mM phosphate buffer, pH 6.8, at 28°. The concentration of papain was 1.1×10^{-5} M. The curves represents the fit of the experimental points to eqn (1). Initial specific activity was 8.1 μ[illegible]/mg protein.

RESULTS AND DISCUSSION

Inactivation of papain by DIMBOA

The semilog plots of the decrease with time of papain activity in the presence of an excess of DIMBOA, gave curves whose slopes increased in a negative sense with time (Fig. 1). This pattern may be interpreted by several kinetic models involving one or more amino acid residues in the inactivation process [12]. Amino acid analysis showed that only cysteine and serine decreased significantly in the modified enzyme [illegible] compared with the

Table 1. Amino acid analysis of papain and papain modified by DIMBOA

Amino acid	Native* papain (ref [13])	Modified* papain	% Change
Try	5	nd†	
Lys	10	10.4	3.8
Arg	12	12	0
Cystine (half)	6	nd†	-
CM-Cys	1	0.5	50
Asp	19	19.6	3.2
Thr	8	7.7	3.8
Ser	13	9.1	30
Glu	20	20	0
Pro	10	9.6	4
Gly	28	27.8	1.3
Ala	14	14.8	5.4
Val	18	16.8	6.6
Ile	12	12	0
Leu	12	11.6	3.3
Tyr	19	19.3	1.6
Phe	4	4.1	2.5

*Residues per mol protein. Data was normalized with respect to arginine and glutamic acid.
†Not detected by this method.

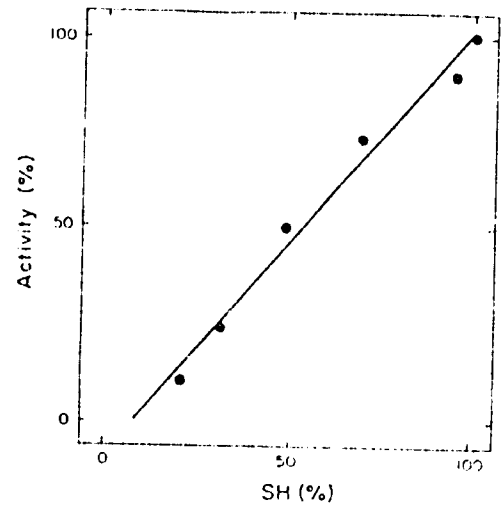


Fig. 2. Proportionality of activity and thiol content of papain as percentage of initial values, during inactivation of the enzyme by DIMBOA. Initial specific activity was 8.9 μmol/min/mg protein, and initial thiol titre was 0.57 mol SH/mol enzyme.

native one (Table 1) [13]. Although tryptophan and cysteine were not detected by this method, these amino acids do not react with DIMBOA in aqueous solution [14]. On the other hand, the ε-amino group of lysine does react with DIMBOA. However, this reaction is negligible at the pH used in the inactivation of papain by DIMBOA [15].

Two models may account for the results. In the first model, the modification of either residue separately will not affect enzyme activity, but modification of both will inactivate the enzyme. In the second model, the modification of one amino acid residue does not affect enzyme activity, but does affect the protein molecule in such a way that a second amino acid residue, essential for enzyme activity, becomes susceptible to inactivator attack. In this latter model, the modification rate of the second amino acid residue must correlate with the loss of enzyme activity.

Papain was incubated with DIMBOA and the thiol titre and activity were determined in parallel. A linear correlation with a slope close to one was obtained between the loss of activity and the loss of thiol titre (Fig. 2), indicating that the second model is consistent with our kinetic data. The equations describing this model are as follows:

$$E + I \xrightarrow{k_1} E_1 + I \xrightarrow{k_2} E_{1,2}$$

$$\frac{E}{E_0} = \frac{k_2}{k_2 - k_1} e^{-k_1 t} - \frac{k_1}{k_2 - k_1} e^{-k_2 t} \qquad (1)$$

The experimental points for the inactivation kinetics were least-squares-fitted to this equation. The theoretical lines generated are shown in Fig. 1. Pseudo-first order rate constants were obtained from the fit. Second order rate constants determined from the slope of a plot of pseudo-first order rate constants versus DIMBOA concentration were $k_1 = 50 \pm 5\ \mathrm{min}^{-1}\ \mathrm{M}^{-1}$ and $k_2 = 10 \pm 2\ \mathrm{min}^{-1}\ \mathrm{M}^{-1}$.

Inactivation of papain by DIMBOA analogues

DIMBOA is known to react with thiols through the reactive aldehyde group of aldol 2, in equilibrium with DIMBOA in solution, or through the hydroxamic nitrogen atom [9]. The reaction of DIMBOA with Cys-25 of papain was determined from studies with DIMBOA analogues 3 and 4. Compound 3 cannot form an open chain tautomer, and hence does not generate a reactive aldehyde group. Compound 4 forms an open chain tautomer with a reactive aldehyde group, but does not have a hydroxamic nitrogen. Inactivation studies with these compounds (50 mM phosphate buffer, pH 6.8) showed that while compound 3 inactivated papain with a kinetic pattern (results not shown) and rate constants ($k_1 = 47 \pm 4\ \mathrm{min}^{-1}\ \mathrm{M}^{-1}$; $k_2 = 9 \pm 1\ \mathrm{min}^{-1}\ \mathrm{M}^{-1}$) similar to that of DIMBOA, compound 4 did not alter papain activity in the same time period.

These results indicate that the reaction of papain with DIMBOA occurs by interaction of the sulphur atom of Cys-25 with the hydroxamic nitrogen of DIMBOA, in a way presumably analogous to the reduction of DIMBOA by thiols [9]. In the non-enzymatic reaction, an intermediate with the sulphur atom bonded to nitrogen reacts with a second thiol molecule to generate a disulphide and the corresponding lactam of DIMBOA [9]. In the reaction of DIMBOA with papain this is unlikely due to steric and concentration effects. Consistent with this proposition, activity was recovered by addition of dithiothreitol (DTT) to inactivated papain (Fig. 3). The DTT added presumably reacted at the sulphur atom of Cys-25 bonded covalently to the hydroxamic nitrogen of DIMBOA, causing the formation of DIMBOA-lactam and a mixed disulphide (enzyme-DTT), which upon reaction with further DTT liberated the active enzyme.

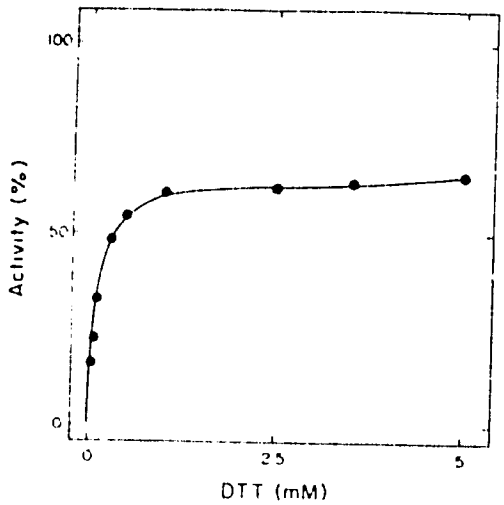


Fig. 3. Recovery of activity by different DTT concentrations of papain inactivated by DIMBOA.

Toxicity of DIMBOA towards insects

DIMBOA is toxic towards several cereal pest insects such as the larvae of the European corn borer, *Ostrinia nubilalis*, and the cereal aphids [1]. The mechanism of toxicity is unknown.

Insect digestive enzymes have been suggested as possible targets for the action of plant defence chemicals [16]. Thiol proteinases have been isolated from the gut of *Callosobruchus maculatus F.* a phytophagous insect [17]. Recently, it was demonstrated that DIMBOA inhibits proteinases isolated from the gut of *Ostrinia nubilalis* [F. Campos, J. Houseman, J. Atkinson and J. T. Arnason, personal communication]. DIMBOA also inactivated α-chymotrypsin [L. Cuevas, H. M. Niemeyer and F. J. Pérez, unpublished work]. Thus, it appears likely that the toxicity of DIMBOA towards phytophagous insects is related to its capacity to inhibit thiol or serine proteinases acting as digestive enzymes.

This proposition is unlikely to explain the toxicity of DIMBOA towards aphids, since aphids are sucking insects which utilize mainly protein-poor plant sap as food source [18]. We were unable to detect serine and thiol proteinase activity in extracts of the cereal aphid *Rhopalosiphum padi*. Acetylcholinesterases in aphids possess a cysteine residue that reacts covalently with thiol reagents leading to enzyme inactivation [19]. DIMBOA inactivated cholinesterase activity from an aqueous extract of the aphid *Rhopalosiphum padi* (results not shown). This may be related to the toxicity of DIMBOA towards aphids. Further studies are underway to test this hypothesis.

EXPERIMENTAL

Enzyme and reagents. Papain (twice recrystallized), *N*-carbobenzyloxyglycine *p*-nitrophenyl ester, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and dithiothreitol (DTT) were purchased from Sigma.

2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (1). This compound was isolated from ethereal extracts of *Zea mays* L. cv. T129s, as described [8].

7-Methoxy-4-hydroxy-1,4-benzoxazin-3-one (3). Nitration of 3-methoxy-phenol followed by reaction with methylbromoacetate and further reductive cyclization, according to the method of ref. [20], afforded this compound. ^{1}H NMR (60 MHz, acetone-d_6, TMS): δ: 3.3 (*s*, 3H), 4.3 (*d*, 2H), 6.7–7 (*m*, 3H), 10.2 (*s*, 1H); EIMS (probe) 70 eV, *m/z* (rel. int.): 195 [M]$^+$ (8), 179 (93), 167 (23), 165 (23), 149 (100), 136 (48).

7-Methoxy-2-hydroxy-1,4-benzoxazin-3-one (4) was synthesized from 2-amino-5-methoxy-phenol and dichloroacetylchloride essentially by the method described in ref. [21]. ^{1}H NMR (60 MHz acetone-d_6, TMS): δ 3.7 (*s*, 3H), 5.5 (*s*, 1H), 6.6–6.9 (*m*, 3H), 8.0 (*s*, H), 10.7 (*s*, 1H); EIMS (probe) 70 eV, *m/z* (rel. int.): 195 [M]$^+$ (42), 166 (100), 150 (13), 138 (25), 124 (40), 110 (46).

Activation of papain. Papain was activated for 30 min at 30° in a soln containing 20 mM cysteine, 50 mM acetate buffer, pH 5.2, and 1 mM EDTA. It was separated from excess activator by filtration through a Sephadex G-25 column equilibrated with the same buffer without cysteine, using a rapid centrifugation-filtration technique [22]. Papain concentration was estimated from the absorption coefficient at 278 nm (ε = 52 000 l/mol cm), and a M_r of 23 700 [13]. Activated papain showed 0.6 mol of SH groups/mol protein, measured according to Ellman's procedure [23]. This figure is in agreement with earlier reports [24].

Assay of papain activity. The hydrolytic activity of papain was measured at 28° with 0.2 mM *N*-carbobenzyloxyglycine *p*-nitrophenyl ester as substrate in 50 mM acetate buffer, pH 5.2. The increase in absorbance at 340 nm due to the release of *p*-nitrophenol was followed [25]. The stock substrate soln (6.1 mM) was prepared in spectroscopic quality MeCN just before use. The MeCN concentration in the assay mixture was 3%. The non-enzymatic hydrolysis of the substrate was taken into account by using substrate in the reference cuvette.

Inactivation of papain by DIMBOA. The reactions were followed under pseudo-first order conditions with an excess of DIMBOA, by taking 0.1 ml aliquots from the reaction mixture (1 ml) after appropriate time intervals and measuring the decrease in the enzymatic activity by the procedure previously described. The dilution of aliquots for the enzymatic assay was at least 30-fold.

Amino acid analysis. A soln (0.5 ml) of 0.5 mM activated papain was filtered through a Sephadex G-25 column equilibrated with 50 mM Pi buffer, pH 6.8. To this soln DIMBOA (0.5 M in 5 μl DMSO) was added, and the mixture incubated for 2 hr at 28°. After this time, papain had lost its activity and was filtered through a Sephadex G-25 column to eliminate the excess of DIMBOA. To this fraction, iodoacetic acid (107 mM, 10 μl) was added and the mixture incubated for 15 min. After this time, the soln was filtered to eliminate the excess of iodoacetic acid, and lyophilysed. The protein was hydrolysed with 6 M HCl at 110° for 20 hr and analysed in a Beckman aminoacid analyzer model 120C.

Titration of the sulphydryl groups in the enzyme. From a reaction mixture (1 ml) containing 0.1 mM gel-filtered activated papain and 20 mM DIMBOA in 50 mM acetate buffer, pH 5, aliquots (0.1 ml) were withdrawn after different time intervals and diluted with 0.7 ml Tris HCl buffer, pH 8. The resulting aliquot was then filtered through a Sephadex G-25 column equilibrated with 0.1 M Tris HCl, pH 8, to eliminate excess DIMBOA before adding the thiol reagent DTNB, since it was found that DIMBOA reacted with it. Protein was determined in each filtrate by its absorption coefficient at 278 nm. A soln of DTNB (0.1 ml, 1 mM) was added, and 5 min later the absorbance was measured at 412 nm (ε = 13 600 l/mol cm). After the same time intervals, 10 μl aliquots were withdrawn and enzymatic activity was assayed according to the standard procedure.

Reversion by dithiothreitol of the inactivation of papain by DIMBOA. A papain soln (1 ml) of specific activity 8.9 μmol/min.mg of protein in 50 mM Pi buffer, pH 6.8, was inactivated with 20 μl of 0.5 M DIMBOA in DMSO. Once the enzyme had lost all its activity, it was filtered through a Sephadex G-25 column to eliminate the excess of DIMBOA. To different aliquots of this enzyme soln, DTT was added in concns ranging from 0.025 to 5 mM, and the kinetics of recovery of activity were followed. Enzyme activity was measured as described previously. Controls were run without the enzyme soln at different DTT concentrations: no release of nitrophenol was observed under the experimental conditions employed.

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