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FINAL REPORT

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ROLE OF HYDROXAMIC ACIDS IN CEREAL-APHID INTERACTIONS

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Granted Institution	:	Facultad de Ciencias Universidad de Chile

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Table of contents

Executive Summary	3
Research Objectives	4
Impact and Relevance of the Project	5
Project Activities/Outputs	6
Publications	6
International meetings	7
National meetings	9
Visitors to the laboratory	10
Latin American fellows trained in the laboratory	11
Chilean students trained in the laboratory	11
Project Productivity	12
Future work	13

Executive summary

Aphids are a major group of insect pests causing severe losses either through direct feeding damage or as a result of transmitting plant viruses. One important management strategy to reduce their impact is to select and breed for resistant plants. At present, this is a long and difficult procedure; however, new technologies in molecular biology hold great promise and will change the way selection procedures are conducted. To take full advantage of these technologies, research on mechanisms of resistance is required and the relationships between gene products, insect fitness and crop damage need to be clearly understood.

This project has examined the role of a group of secondary chemicals in wheat (hydroxamic acids - Hx) as aphid resistant factors in the plant. Specific objectives have been to evaluate antixenosis of Hx towards aphids; to determine the effect of Hx on transmission of barley yellow dwarf virus by aphids, to study the effects of Hx on aphid parasitoids and predators, to describe ways of increasing Hx concentrations in wheat, and to explore mechanisms of toxicity of Hx.

We have shown that Hx are feeding deterrents towards aphids; that virus transmission by aphids is decreased by Hx in the plant; that high levels of Hx in wheat plants have a favorable effect on a main aphid predator; that germplasm leading to high Hx levels in the plant exists among close relatives of wheat; and that the toxicity of Hx may be due to the inhibition of key metabolic enzymes.

The results of this project have given rise to 22 publications in international journals, and have been presented at 10 international meetings. As a result of this project, 15 graduate and undergraduate students have been trained, and the laboratory is conditioned to carry out in a modern way research in a wide range of topics related to Entomology, Biochemistry and Chemistry, and thus to approach agricultural problems in a multidisciplinary way.

Research Objectives

Two main components of integrated pest management are host lant resistance and biological control. However, the increase of atural resistance of crops to pests, for example through an ncrease in the concentration of plant secondary metabolites hich may act as defensive compounds, is not devoid of risks due o possible effects at other levels in the food chain. dditionally, the efficient manipulation of the concentration of secondary metabolite rests on knowledge of its mechanism of ction. In this project, the role of hydroxamic acids in the efense of wheat against aphids was examined, their effect on opulations of an important aphid predator determined and their hemical mechanism of toxicity explored.

Research efforts on wheat resistance to barley yellow dwarf irus are mainly directed towards finding specific resistance in he infected plant. In this project emphasis was shifted to the ontrol of virus transmission through the antibiotic and ntixenotic effects of hydroxamic acids on the virus vector.

Breeding programs for resistance are normally based on time-onsuming and costly infestation ratings. In this project, vidence was sought for the use of hydroxamic acids as markers or aphid resistance in wheat and for the existance of suitable arental germplasm for breeding.

Several organizations have been involved in providing upport complementary to that granted by US-AID. The nternational Program in the Chemical Sciences at the University f Uppsala, Sweden (IPICS) has provided research funds and funds o launch a training program in Ecological Chemistry in Latin merica. The International Foundation for Science (IFS) has rovided research grants to staff members and graduate students n the laboratory. The British Council (BC) has provided funds or visits by Chilean scientists to the UK and by British cientists to Chile.

Impact and Relevance of the Project

During the past few years there has been a continued decrease in the relative importance of aphids among the factors decreasing wheat yield in Chile. Wheat breeders in Chile, although supportive of our work, have not become directly involved in it. However, since little is known about factors other than biological control affecting aphid populations in Chile, the work is seen as an important contribution towards understanding aphid-cereal interactions and thus towards being prepared for undesirable aphid population explosions. The appearance of the Russian Wheat Aphid in Chile has produced much local interest in our project. Additionally, our work has called the attention of agronomists who realize the potential of examining other insect-plant interactions from a multidisciplinary viewpoint.

The present AID project has permitted the development of a laboratory with reasonable infrastructure, with capacity to work in a wide range of scientific disciplines, and very attractive to Ph.D. candidates with varying academic backgrounds.

The impact in other parts of the world has been considerable. Laboratories in England (University of Southampton, Rothamsted Experimental Station) and in Sweden (Swedish University of Agricultural Sciences), as well as breeding companies (Svalöv AB in Sweden) have began projects, mostly in conjunction with our research group, to investigate further the potential of hydroxamic acids as resistance factors in wheat. Our laboratory has received the visits of numerous scientists for periods ranging from days to months.

Additionally, mainly with the support of IPICS, the research philosophy of our laboratory is being disseminated towards Latin America through a training program in Ecological Chemistry. Our laboratory has received the visits of several trainees from Latin America.

Project Activities/Outputs

The productivity of our laboratory may be judged by the publications in scientific journals or monographies, the presentation of papers at international and national meetings, by the visitors the laboratory has received, by the Latin American fellows that have received training in it and by the Chilean students and technicians trained in it.

Publications

1. Pérez, F.J. and Niemeyer, H.M. (1989) Reaction of DIMBOA, a Resistance Factor From Cereals, with Papain. **Phytochemistry** 28: 1597-1600.

2. Niemeyer, H.M. (1990) The Role of Secondary Plant Compounds in Aphid-Host Interactions, in **Aphid-Plant Genotype Interactions**, Campbell, R.K. and Eikenbary, R.D., eds., pp. 187-205.

3. Thackray, D.J., Wratten, S.D., Edwards, P.J. and Niemeyer, H.M. (1990) Resistance to the Aphids Sitobion avenae and Rhopalosiphum padi in Gramineae in Relation to Hydroxamic Acid Levels. **Annals of Applied Biology** 116: 573-582.

4. Pérez, F.J (1990) Allelopathic effect of hydroxamic acids from cereals on Avena sativa and A. fatua. **Phytochemistry** 29: 773-776.

5. Cuevas, L., Niemeyer, H.M. and Pérez, F.J. (1990) Reaction of DIMBOA, a Resistance Factor From Cereals, with α-Chymotrypsin. **Phytochemistry** 29: 1429-1432.

6. Pérez, F.J. and Ormeño-Núñez, J. (1990) Root exudates of wild oats (Avena fatua L.): allelopathic affect on Spring wheat (Triticum aestivum L.). **Phytochemistry** 30: 2199-2202.

7. Givovich, A. and Niemeyer, H.M. (1991) Hydroxamic Acids Affecting Barley Yellow Dwarf Virus Transmission by the Aphid Rhopalosiphum padi. **Entomologia Experimentalis et Applicata** 59: 79-85.

8. Wratten, S.D., Martin, J.J., Rhind, D. and Niemeyer, H.M. (1991) Mechanisms of Resistance in Cereals to Pests. **Proceedings of 1990 Brighton Pest Control Conference-Pests and Diseases-1990**. pp. 925-934.

9. Pérez, F.J. and Ormeño-Núñez, J. (1991) Difference in hydroxamic acid content in roots and root exudates of wheat (Triticum aestivum L.) and rye (Secale cereale L.): possible role in allelopathy. **Journal of Chemical Ecology** 17: 1037-1043.

10. Niemeyer, H.M. (1991) Secondary Plant Chemicals in Aphid-Host Interactions. **Aphid-plant interactions: populations to molecules**. Peters, D.C., Webster, J.A. and Chlouber, C.S., eds., USDA/Agricultural Research Service, Oklahoma State University. pp. 101-111.

11. Copaja, S.V., Barría, B.N. and Niemeyer, H.M. (1991) Hydroxamic Acid Content of Perennial Triticeae. **Phytochemistry** 30: 1531-1534.

12. Copaja, S.V., Niemeyer, H.M. and Wratten, S.D. (1991) Hydroxamic Acid Levels in Chilean and British Wheat Seedlings. **Annals of Applied Biology** 118: 223-227.

13. Barría, B.N., Copaja, S.V. and Niemeyer, H.M. (1991) Occurrence of DIBOA in Wild Hordeum Species and Relation to Aphid Resistance. **Phytochemistry**, in press.

14. Bravo, H.R. and Niemeyer, H.M. (1991) Decomposition of 7-Nitro-2,4-dihydroxy-1,4-benzoxazolin-3-one in Aqueous Solutions. **Heterocycles**, in press.

15. Quiroz, A. and Niemeyer, H.M. (1991) Reaction of 7-Substituted-4-hydroxyl-1,4-benzoxazin-3-ones in Strongly Acidic Media. **Heterocycles**, in press.

16. Niemeyer, H.M., Copaja, S.V. and Barría, B.N. (1992) Sources of Hydroxamic Acids, Secondary Metabolites in Wheat Conferring Resistance Against Aphids. **Hereditas**, submitted.

17. Martos, A., Givovich, A. and Niemeyer, H.M. (1992) Effect of DIMBOA, an Aphid Resistance Factor in Wheat, on the Aphid Predator Eriopis connexa Germar (Col.: Coccinellidae). **Journal of Chemical Ecology**, submitted.

18. Givovich, A., Morse, S., Cerda, H., Niemeyer, H.M., Wratten, S.D. and Edwards, P.J. (1992) Hydroxamic Acid Glucosides in the Honeydew of Aphids Feeding on Cereals, and Their Relation to Aphid Growth Rates. **Journal of Chemical Ecology**, submitted.

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.

2. Niemeyer, H.M., Pérez, F.J., and Cuevas, L. (1989) Chemical Basis for Toxicity of Hydroxamic Acids from Cereals. **32nd IUPAC Congress**, Stockholm, Sweden.

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 Ecolcgy**, Gothemburg, Sweden.

4. Pérez, F.J. and González, L.F. (1990) Auxin high affinity binding sites from coleoptile membranes of avena (Avena sativa L.). **7th Congress of the Federation of European Societies of Plant Physiologists**. Umeå, Sweden.

5. Niemeyer, H.M. (1990) Secondary Plant Chemicals in Aphid-Host Interactions. **International Symposium on Aphid-plant interactions: populations to molecules**. Stillwater, Oklahoma, U.S.A..

6. Wratten, S.D., Thackray, D.J., Niemeyer, H.M., Morse, S. and Edwards, P.J. (1990) The Role of Hydroxamic Acids in Wheat and Maize in Conferring Resistance to Aphids. **International Symposium on Aphid-plant interactions: populations to molecules**. Stillwater, Oklahoma, U.S.A..

7. Wratten, S.D., Martin, J.J., Rhind, D. and Niemeyer, H.M. (1990) Mechanisms of Resistance in Cereals to Pests. **Brighton Pest Control Conference, Pests and Diseases**, Brighton, England.

8. Thackray, D.J., Wratten, S.D., Edwards, P.J. and Niemeyer, H.M. (1990) Hydroxamic Acids - Potential Resistance Factors in Wheat Against the Cereal Aphids Sitobion avenae and Rhopalosiphum padi. **Brighton Pest Control Conference, Pests and Diseases**, Brighton, England.

9. Givovich, A. (1990) Hydroxamic acids affecting Barley Yellow Dwarf Virus transmission by aphids. **IFS Regional Meeting on Plant Viruses**, Argentina.

10. Copaja, S.V. (1990) Producción de ácidos hidroxámicos en plantas de trigo. **III Congreso Latinoamericano de Cromatografía**. Aguas de San Pedro, Brazil.

11. Copaja, S.V. and Barría, B.N. (1990) Acidos hidroxámicos en la quimiotaxonomía de gramíneas silvestres. **XIX Congreso Latinoamericano de Química**. Buenos Aires, Argentina.

12. Niemeyer, H.M., Copaja, S.V. and Barría, B.N. (1991) Perennial Triticeae as Source of Hydroxamic Acids, a Resistance Factor in Cereals. **First International Triticeae Symposium**, Helsingborg, Sweden.

National meetings

1. Givovich, A. (1989) Feeding preferences and behavior of Rhopalosiphum padi L. in wheat with different hydroxamic acid levels. **IFS Workshop on Chemical Interaction Between Organisms**, Santiago, Chile.

2. Copaja, S.V. and Barría, B.N. (1989) Hydroxamic acid content in perennial Triticeae. **IFS Workshop on Chemical Interactions Between Organisms**, Santiago, Chile.

3. Cuevas, L. (1989) Characterization of cholinesterases from Rhopalosiphum padi and their reaction with DIMBOA. **Workshop on Chemical Interactions Between Organisms**, Santiago, Chile.

4. Bravo, H.R. (1989) Chemical reactivity of hydroxamic acids from cereals. **IFS Workshop on Chemical Interactions Between Organisms**, Santiago, Chile.

5. Pérez, F.J. and L.F. González (1989) Allelopathic effect of hydroxamic acids from wheat on oat Avena sativa L. and on wild oat Avena fatua L. **IFS Workshop on Chemical Interactions Between Organisms**, Santiago, Chile.

6. Martos, A. (1989) Effect of DIMBOA on development of Eriopis connexa Germar (Col.: Coccinellidae). **IFS Workshop on Chemical Interactions Between Organisms**, Santiago, Chile.

7. Quiroz, A. (1989) Behavior of 4-hydroxy-1,4-benzoxazin-3-ones in strong acic media. **IFS Workshop on Chemical Interactions Between Organisms**. Santiago, Chile.

8 Copaja, S.V. and Barría, B.N. (1989) Contenido de ácidos hidroxámicos en gramíneas silvestres. **XVIII Jornadas Chilenas de Química**, Santiago, Chile.

9. Weiss, B. and Bravo, H.R. (1989) Estudio conformacional de 2,4-dihidroxi-7-metoxi-1,4-benzoxazin-3-ona (DIMBOA). **XVIII Jornadas Chilenas de Química**, Santiago, Chile.

10. Bravo, H.R. (1989) Efecto del sustituyente en la descomposición de ácidos hidroxámicos cíclicos de cereales. **XVIII Jornadas Chilenas de Química**, Santiago, Chile.

Visitors to the laboratory

Prof. J. T. Arnason, University of Ottawa, Canada, August 1988

Prof. B.J.R. Philogène, University of Ottawa, Canada, August 1988

Dr. J. Atkinson, University of Ottawa, Canada, October 1988

Prof. J. R. Cannon, University of Western Australia, Ausralia, November 1988

Prof. J. L. Auclair, University of Montréal, Canada, March-April 1989

Prof. C. A. Oehschlager, University of British Columbia, Canada, November 1989

Prof. G. D. Prestwich, State University of New York, USA, November 1989

Prof. F. A. Einhellig, University of South Dakota, USA, November 1989

Prof. K. S. Brown, Campinas State University, Brazil, November 1989

Prof. T. Norin, Royal Institute of Technology, Sweden, November 1989

Prof. J. Pettersson, Swedish University of Agricultural Sciences, Sweden, November 1989

Prof. P. Baeckström, Royal Institute of Technology, Sweden, November 1989

Dr. S.D. Wratten, Southamton University, England, November 1989, September 1991

Ms. M. Friberg, Pharmacia AB, Sweden, March 1990

Dr. W. F. Tjallingii, Wageningen Agricultural University, The Netherlands, April 1990

Dr. L. Jonsson, Swedish University of Agricultural Sciences, Sweden, April 1990

Dr. F. Dajas, Instituto de Investigaciones Biológicas Clemente Estable, Uruguay, June 1990, July 1991

Dr. P. J. Edwards, Southampton University, England, October 1990

Prof. Michael Wink, University of Heidelberg, Germany, December 1990

Latin American fellows trained in the laboratory

Jairo Calle, Universidad Nacional de Colombia, September-December 1989

Augusto Rivera, Universidad Nacional de Colombia, September-December 1989

Agustín Martos, Universidad Nacional Agraria La Molina, Peru, September 1989- January 1990

Gabriela Manrique, Internmational Potato Center, Peru, July-October 1990

Hugo Cerda, Universidad Simón Bolívar, Venezuela, September-December 1990

Ricardo Reátegui, Universidad Nacional Agraria La Molina, Peru, September 1990 - June 1991

Alba Arango, Universidad Nacional de Colombia, April 1991-February 1992

Bárbara Moreno, Universidad Nacional de Colombia, July 1991-May 1992

Chilean students trained in the laboratory

Ph.D. students

Héctor Asencio (Biochemistry, year 2)
Bernardita Barría (Chemistry, year 2)
Susana Casas (Chemistry, year 2)
Arturo Givovich (Chemistry, year 3)
Victoria Leighton (Biochemistry, year 3)
Andrés Quiroz (Chemistry, year 1)

M.Sc. students

Liliana Cuevas (Biochemistry)
María Isabel Olmedo (Genetics)
Andrés Quiroz (Chemistry)

Undergraduate students

Bernardita Barría (Chemistry)
Sandra Carter (Chemistry)
Carlos Escobar (Chemistry)
César González (Chemistry)
Cristina Pedraza (Chemistry)
Ximena Tapia (Chemistry)

Technicians

Paola Bravo (Chemistry)
Lucía Muñoz (Entomology)
Patricia Muñoz (Botany)
Claudia Ocares (Chemistry)
Luisa Ramírez (Chemistry)
Allison Soriano (Entomology)

Project Productivity

The coverage of the areas described in the proposal has been uneven. Most efforts have been directed towards understanding the effect of hydroxamic acids in the transmission of virus by aphids, in describing the effect of hydroxamic acids on an aphid predator, and in finding germplasm leading to the accumulation of high levels of hydroxamic acids in graminaceous plants.

While work is still in progress in the area of environmental effects on the accumulation of hydroxamic acids in wheat, the effect of Hx on aphid parasitoids has still not been addressed.

On the other hand, a new area which was judged promising was included. It is the case of allelopathy by Hx, described below.

Allelopathy by hydroxamic acids

Weeds constitute one of the mejor sources of yield decreases in crops. Losses are important and the cost of herbicides considerable. These facts, together with high concern developed lately towards the environment, have stimulated an academic and comercial interest in a biotechnological approach to weed control. The subject of chemical and biochemical interactions between plants (allelopathy) is an area of great relevance for weed control. The capacity of crop plants to produce and release chemicals inhibiting the development of weeds provides the plant with natural herbicides which could be exploited in the benefit of the crop plant.

Research is this area has been developed in close contact with weed scientists at Instituto de Investigaciones Agropecuarias (INIA). The emphasis is on the use of hydroxamic acids from cereals in the control of weeds of those crops.

Hydroxamic acids were shown to be toxic to a wide range of weeds in concentrations not affecting the crop plant. Roots of cereals contained hydroxamic acids but, with the exception of a rye cultivar, the compounds were not exuded to the environment. Field studies showed that this rye cultivar had a high capacity to supress weeds. The nature of allelopathic compounds in wild oats was also shown.

Future work

In the area of wheat-aphid interactions, the following problems should be addressed:

1) evaluation of the effect of hydroxamic acids on aphid parasitoids.

2) biosynthesis of hydroxamic acids and to characterization of the genes involved in their accumulation.

3) cultural conditions maximizing the accumulation of hydroxamic acids in wheat plants.

4) origin of the toxicity and feeding deterrency of hydroxamic acids and effect of them on aphid enzymatic systems.

5) breeding aimed at increasing hydroxamic acid levels in wheat plants.

In the area of allelopathic effects of hydroxamic acids in cereals, the following problems should be addressed:

1) evaluation of the weed-suppressing capacity of rye cultivars exudating hydroxamic acids, and relationship of this capacity to the ability to exudate hydroxamic acids.

2) existance of wheat and rye germplasm with capacity to exudate hydroxamic acids.

3) breeding in rye incorporating the capacity to exudate hydroxamic acids into agronomically useful cultivars.

4) mechanism of exudation of hydroxamic acids through the roots and the factors that control the process, and possibility to transfer these properties to crops capable of producing hydroxamic acids but incapable of exudating them.

5) molecular mechanisms of inhibition by hydroxamic acids of root and coleoptile growth in avena.

Financial report

Equipment (all itmes in the inventory of Universidad de Chile)

Centrifuge (Beckman)		18,692.40
Oven (Memmert)		2,215.00
Ice maker (Scotman)		2,125.00
Water purifier (Millipore)		2,910.00
Microscope, stereoscope (Nikon)		2,189.14
Sterilizer (VWR)		765.00
Plant growth chamber (Norstream)		15,832.06
Vacuum valves (MKS)		1,252.25
Radioactivity flow detector (Beckman)		16,574.00
Refrigerator (Fensa)		559.67
UV Sample injector (Shimadzu)		3,648.28
Customs, duties and taxes		6,386.30
S u b t o t a l	**US$**	**73,149.10**

Supplies

Chemicals (Sigma Chem. Co.)		8,634.38
Radiochemicals (Farmanuclear)		1,321.60
Labware and glassware		7,995.07
S u b t o t a l	**US$**	**17,951.05**

Salaries

Graduate students

Liliana Cuevas		2,998.04
Arturo Givovich		8,181.69
Victoria Leighton		6,677.10
Isabel Olmedo		1,650.00
Andrés Quiroz		3,419.05

Technicians

Tito Acuña		236.22
Paola Bravo		1,017.29
Ana Flores		2,666.70
Lucía Muñoz		1,475.10
Patricia Muñoz		1,959.13
Claudia Ocares		2,073.42
Luisa Ramírez		2,029.33
S u b t o t a l	**US$**	**34,383.07**

Travel (Hermann M. Niemeyer) **US$ 12,970.00**

Other costs (computer accessories, reprints, books, miscellaneous lab aids) **US$ 10,546.78**

Budget summary

Item	Requested	Spent
Equipment	US$ 76,500	US$ 73,149.10
Supplies	19,000	17,951.05
Salaries	31,500	34,383.07
Travel	13,000	12,970.00
Other costs	9,000	10,546.78
T o t a l	**US$ 149,000**	**US$ 149,000.00**

Hermann M. Niemeyer
Principal Investigator

Carlos Andrade Plaza
Dean

Juan Carlos Hidalgo
Imports Officer

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

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(*Received in revised form* 18 *November* 1988)

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

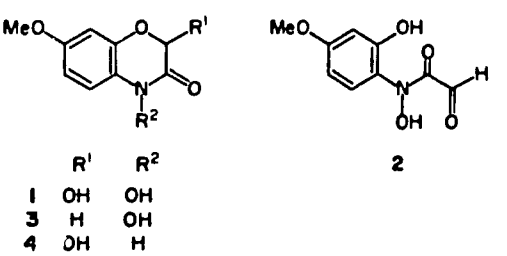
Abstract—2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), a hydroxamic acid from cereals involved in host plant resistance to insects, inactivated papain (EC 3.4.2.2). Semilog plots of the residual activity of papain in the presence of an excess of DIMBOA as a function of time were concave downwards. 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.



	R'	R²
1	OH	OH
3	H	OH
4	OH	H

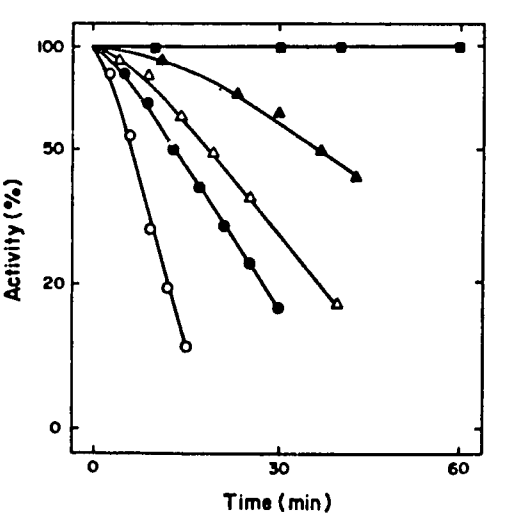


Fig. 1. Kinetics of papain inactivation at different DIMBOA concentrations: 0 (■), 3 (▲), 5 (△), 8 (●), and 15 mM (○), in 50 mM phosphate buffer, pH 6.8, at 28°. The concentration of papain was 1.1×10^{-5} M. The curves represents the fit of the experimental points to eqn (1). Initial specific activity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

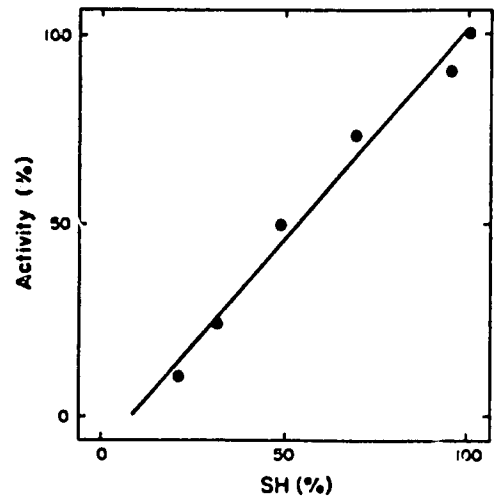


Fig. 2. Proportionality of activity and thiol content of papain as percentage of initial values, during inactivation of the enzyme by DIMBOA. Initial specific activity was 8.9 μmol/min/mg protein, and initial thiol titre was 0.57 mol SH/mol enzyme.

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:

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

$$\frac{E}{E_0}=\frac{k_2}{k_2-k_1}e^{-k_1t}-\frac{k_1}{k_2-k_1}e^{-k_2t} \qquad (1)$$

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 $k_1=50\pm5\ \mathrm{min^{-1}\,M^{-1}}$ and $k_2=10\pm2\ \mathrm{min^{-1}\,M^{-1}}$.

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 ($k_1=47\pm4\ \mathrm{min^{-1}\,M^{-1}}$; $k_2=9\pm1\ \mathrm{min^{-1}\,M^{-1}}$) 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.

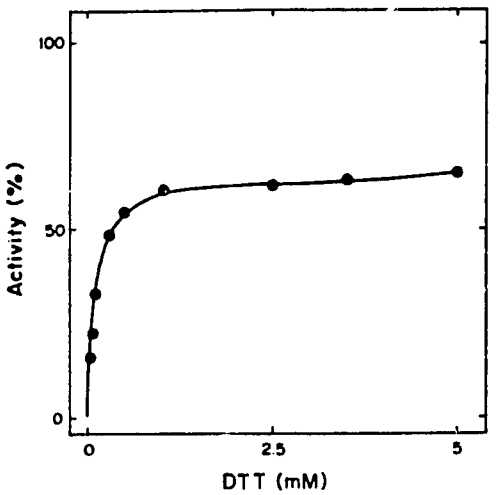


Fig. 3. Recovery of activity by different DTT concentrations of papain inactivated by DIMBOA.

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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THE ROLE OF SECONDARY PLANT COMPOUNDS IN APHID-HOST INTERACTIONS

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1. Introduction
2. Landing
3. Testing of plant surface and outer plant tissues
4. Penetration and feeding
   4.1 Alkaloids
   4.2 Phenolic compounds
   4.3 Glucosinolates
   4.4 Hydroxamic acids
   4.5 Other compounds
5. Conclusions

1. INTRODUCTION

The settling of an aphid on a proper host plant results from a sequence of steps which may be summarized as follows: 1) landing, 2) testing of the plant surface and the outer plant tissues, and 3) penetration and evaluation of the final feeding tissues (Hille Ris Lambers, 1979; Klingauf, 1987). The relative importance of these steps varies among different morphs of an aphid, and are dependent on factors such as environmental conditions and morphological and chemical features of the plant.

In this chapter, stimuli from secondary metabolites in the plant influencing aphid host-finding and feeding behaviour are examined.

2. LANDING

Evidence has accumulated on the importance of olfaction of plant volatiles in the orientation of an aphid to its host plant. Olfaction is performed by sensory organs located in the antennae. While their low number suggests that olfaction may become important only at short range (Anderson and Bromley, 1987), their distribution in different aphid morphs suggests the importance of olfaction in migration (Dunn, 1978; Shambaugh, 1978; Bromley et al., 1979; Bromley and Anderson, 1982). Two primary rhinaria in the two distal segments of the antennae are composed of placoid

sensilla structured as typical olfactory receptors. They are present in all instars and although fairly non-specific as receptors, they are the main receptors for an aphid alarm pheromone (E)-β-farnesene (Nault et al., 1973; Wohlers and Tjallingii, 1983) and also for plant volatiles (Pettersson, 1970a). Secondary rhinaria are only present in adults, in greater number in alate males and females, and in apterous males. They may be altogether lacking in apterous females of certain species. While in the males the secondary rhinaria are responsible for detection of the sex pheromone (Pettersson, 1970b), in alate females they have been associated with host selection.

Electrophysiological recordings of antennal receptors of Nasonovia ribis-nigri Mosley (Bromley and Anderson, 1982) and Sitobion avenae Fabrici (Yan and Visser, 1982), revealed their responses to various plant volatile compounds.

Using an olfactometer of simple design, it was shown that gynoparae and males of Rhopalosiphum padi L. were attracted to extracts of buds of Prunus padus, their primary host (Pettersson, 1970a). Alatae of non-migratory generations were not attracted (Petersson, 1973). The receptors involved were shown to be the primary rhinaria. It was suggested that the principal compound involved in the attraction was benzaldehyde, arising from the enzymatic hydrolysis of prunasine, a glycoside characteristic of Prunus, followed by the decomposition of the aglycone. The importance of olfaction as opposed to tasting in host selection by gynoparae of R. padi was further supported by their non-random landing pattern on P. padus (Leather, 1986), in spite of the fact that they do not feed as adults (Leather, 1982; Walters et al., 1984).

Similar olfactometer experiments carried out with alate virginoparae of Brevicoryne brassicae L. showed that they were attracted to the scent of flowers and buds of Brassica plants, possibly in connection with higher concentrations of sinigrin and CO_2 (Pettersson, 1973).

Using an olfactometer of different design, it was shown that alate Aphis gossypii Glover were attracted selectively by the scent of their host plants Cucurbita pepo and Thunbergia laurifolia while being repelled by the scent of the non-host

The orientation responses of Cryptomyzus korschelti Borner were studied using a locomotion compensator in front of a wind tunnel (Visser and Taanman, 1987). While still air, wind, and wind carrying the odour of the non-host plant Solanum tuberosum induced tortuous tracks, wind carrying the odour of the host plant Stachys sylvatica provoked a clear up-wind orientation, providing evidence for host plant odour-conditioned positive anemotaxis.

Attraction of flying Cavariella aegopodii Scopoli to traps baited with plant chemicals was demonstrated in field experiments (Chapman et al., 1981). Carvone, a compound frequently found in the umbelliferous summer hosts of C. aegopodii, was the most attractive compound tested.

On the other hand, evidence for olfactory reactions could not be proved during host finding by adult apterous Myzus persicae Sulzer or grass-colonizing virginoparae of R. padi (Ahman et al., 1985), nor in the orientation of apterous M. persicae to target hosts in an artificial arena (Hodgson and Elbakhiet, 1985).

The data accumulated so far is still scarce, but there is indication that monophagous morphs of aphids tend to rely more heavily on olfactory cues than on visual cues for host finding. The inverse tendency would seem to prevail in polyphagous morphs. It is interesting to note in this context that aphids with specialized feeding behavior tend to develop annular rhinaria, an adaptation ideally suited for host finding without tasting (Shaposhnikov, 1987).

3. TESTING OF PLANT SURFACE AND OUTER PLANT TISSUES

Once an aphid has landed on a plant, it explores the plant surface with the antennae and engages in brief probes. This behavior is associated with the testing of the chemical nature of the surface and outer tissues of the plant.

Chemoreceptors are present in the antennae in the tip of the flagellum and sometimes lower down on the processus terminalis (Bromley et al., 1979). Sensilla located on the apex of the tibiae and the tarsi may also have a gustatory function (Anderson and Bromley, 1987). It should be noted that the short hairs or pegs on the tip of the labia, which were thought to be chemoreceptors, have been shown to correspond to mechanoreceptors (Wensler, 1977; Tjallingii, 1978).

The importance of surface testing was suggested by experiments with M. persicae males. They preferred to feed and tostay longer on peach (host) twigs than on pear, apricot or apple (non-host) twigs, and coating apple twigs with an extract of peach buds made them more attractive than untreated apple twigs (Tamaki et al., 1970).

Leaf epicutilar waxes were shown to be important in host selection by Acyrthosiphon pisum Harris (Klingauf, 1971, 1975; Klingauf et al., 1978). Wax fractions of the host Vicia faba but not of the non-host Brassica napus stimulated walking of the aphid towards its feeding site. Mechanical destruction of the wax layer or inhibition of its biosynthesis increased the time needed for the aphid to find its feeding place on the host plant (Klingauf et al., 1978). Longer initial probes were obtained when non-host disks were treated with host wax extracts (Klingauf, 1975). Alkanes constituted the most active fractions (Klingauf et al., 1978).

The higher proportion of diketones in the wax of some wheat cultivars increased infestation by S. avenae (Lowe et al., 1985). The origin of the effect was not determined.

Leaf surface extracts of V. fabae and Beta vulgaris, host plants of the aphid Aphis fabae Scopoli, proved attractive to the aphid when added to an artificial diet. Low molecular weight phenols in the extract exhibited highest attractiveness, suggesting they may play a role in host selection (Jordens-Rottger, 1979).

Other surface chemicals which an aphid encounters while walking and probing are the secretions in the trichomes of some plants. Toxic alkaloids, the major one being nicotine, were isolated from the trichome secretions of several Nicotiana species. Contact with these secretions caused acute toxicity in Rhopalosiphum maidis Fitch and M. persicae (Thurston et al., 1966). As the plant aged, the amount of trichome exudates increased and toxicity to M. persicae increased concomitantly (Abernathy and Thurston, 1969). Trichome exudates of the wild tomato Lycopersicon hirsutum f. glabratum contain 2-tridecanone, shown to be toxic to A. gossypii (Williams et al., 1980).

Two types of foliar glandular trichomes may be found in potato plants: type A with a tetralobulate gland at its apex, and type B with an ovoid gland at its tip (Tingey, 1985). Trichomes of type B in the wild potato Solanum berthaulti contain the aphid alarm pheromone (E)-β-farnesene, which is released in amounts capable of repelling the aphid M. persicae at a close range (Gibson and Pickett, 1983). Removal of droplets from trichomes resulted in a decrease in preprobe time by M. persicae compared with that on intact leaves (Lapointe and Tingey, 1984). Transfer of droplets to leaves of domestic potato S. tuberosum, lead to fewer probes, increased time to first probe and reduced total feeding activity compared with untreated leaves (Lapointe and Tingey, 1984). Furthermore, removal of exudates from trichomes of another wild potato species, S. neocardenasii, lead to a decrease in the length of developmental period and an increase in survival of M. persicae (Lapointe and Tingey, 1986).

Recently, trichomes of type A were also shown to contain (E)-β-farnesene. However, they did not hinder aphid attack, presumably due to the difficult detachment of the tetralobulate gland from the trichome stalk (Avé et al., 1987).

(E)-β-farnesene was also found in trichomes of S. tuberosum but did not show a repellent effect. This was attributed to the simultaneous presence of the aphid alarm pheromone inhibitor, (-)-β-caryophyllene (Avé et al., 1987). Similar effects had been observed in relation to the lupulin gland contents in hops Humulus lupulus (Dawson et al., 1984).

In view of the results presented, the importance of surface chemicals in the interaction between an aphid and a plant must be recognized. It is an area which deserves closer attention by both plant anatomists and chemists.

4. PENETRATION AND FEEDING

The final acceptance of a plant by an aphid is determined by the tasting of internal components. Gustation of ingesta is carried out by chemosensory structures in the food canal: an epipharyngeal organ possessing gustatory papillae has been described in B. brassicae and Tuberolachnus salignus Gmelin (Wensler and Filshie, 1969), and in A. pisum (Mc Lean and Kinsey, 1985).

The quality and quantity of plant compounds encountered by a feeding aphid depends on the path followed by the stylet on its way to the final feeding site, on the ingestion of plant

constituents during penetration and on the nature of the final feeding site.

While most aphids are phloem feeders, some feed in xylem or in mesophyll tissue (Pollard, 1973). The path followed by the stylets may be intracellular, intercellular or intramural (Kimmins, 1986). Recent studies indicate that intracellular pathways are more frequent than realized before (Spiller et al., 1985; Al-Mousawi et al., 1983; Tjallingii, 1985). There is also an indication that ingestion occurs before the aphid stylet has reached the final feeding site (Bradley, 1952; McLean and Kinsey, 1967; Lowe, 1967a,b; Henning, 1968; Scheller and Shukle, 1986; Brzezina et al., 1986). Thus, host discrimination may occur very early in the feeding approach (Adams and Wade, 1976).

Hence, although aphids tend to be selective in their feeding site, and thus be able to avoid toxic substances in certain tissues of the plant (Guthrie et al., 1962; Montgomery and Arn, 1974), the intake or contact with substances outside the vessels where they feed can not be dismissed a priori.

4.1. Alkaloids

Quinolizidine alkaloids produced by Lupinus spp. and other plant species are important in the interaction of legume feeding aphids with the plant. Polyphagous aphids, such as A. pisum, are able to colonize only sweet (alkaloid poor) lupines. The susceptibility of these hosts was reduced by infiltration of the alkaloids into them (Wegorek and Krzymanska, 1968, 1971). Some aphids however, are able to colonize alkaloid-rich plants. Acyrthosiphon spartii Koch lives on the sparteine rich broom Sarothamnus scoparius. The aphid migrates within developing plants following sparteine rich tissue. Sparteine was shown to be a feeding stimulant (Smith, 1966).

Aphis cytisorum lives on Lupinus and Cytisus species having relatively high alkaloid content. Alkaloids were found in the phloem of the plant (Wink et al., 1982; Wink and Witte, 1984). Aphids accumulated alkaloids. The composition of the alkaloid mixture in aphids was similar, but not the same, to that found in the plants (Wink et al., 1982; Wink and Witte, 1985).

Another aphid species which has specialized in feeding in certain alkaloid-rich plants is Macrosiphon albifrons Essig. The aphid does not infest alkaloid-free plants, and qualitative

alkaloid patterns as well as alkaloid content are important in determining the infestation levels of alkaloid-containing plants. Although the alkaloids ingested were found in the honeydew, aphids accumulated them in concentrations that proved toxic to predators (Wink and Romer, 1986).

Indole alkaloids, such as gramine, produced by Hordeum species, are important in the interaction of aphids with the plants. Infestation by the aphids Schizaphis graminum Rondani (Zúñiga et al., 1985) and R. padi (Zúñiga and Corcuera, 1986) were inversely correlated with gramine levels in barley plants. Older plants with lower gramine levels were more susceptible to infestation than younger ones. Gramine reduced survival and reproduction rate of both aphid species when added to artificial diets. Since gramine was not found in the vascular bundles, it was hypothesized that it could be encountered by aphids during their search for phloem tissue (Argandoña et al., 1987).

4.2. Phenolic compounds

Differences in ability to colonize different apple varieties and different parts of any one variety by the wooly aphid Eriosoma lanigerum Hausmann was found to be related to content of phenolics and α-amino nitrogen compounds. An inverse linear correlation was obtained between susceptibility and ratio of phenolics to α-amino nitrogen (Sen Gupta and Miles, 1975). This correlation was suggested to arise from the influence of the nutritional status of the plant on the ingestion of toxic compounds (Sen Gupta and Miles, 1975).

The phenolic compound phlorizin, characteristic of the apple genus, was found to be neutral as a probing stimulus to Aphis pomi DeGeer, an apple feeding aphid, but was a probing deterrent to the non-apple feeding aphids, M. persicae and Amphorophora agathonica Hottes. Phlorizin was an ingestion deterrent to the three although the threshold was lower for A. pomi. The fact that apple is utilized as a host by A. pomi was associated with its feeding in the phloem, which appeared not to contain phlorizin (Montgomery and Arn, 1974).

The phenolic compound catechin was suggested as a factor in rose buds whose concentration increase as the sepals open, induces the aphid Macrosiphum rosae L. to walk off them (Miles, 1985).

Phenolic compounds play an important role in habitat selection by the galling aphid Pemphigus betae Auct. (Zucker, 1982). Inverse correlations were found between total phenols and suitability of a tree, leaf or leaf section for galling at the time of bud burst in Populus augustifolia. The findings were consistent with the known biology and territorial behaviour of the aphids (Zucker, 1982).

4.3. Glucosinolates

Glucosinolates are important in host selection by the aphid B. brassicae. When the glucosinolate sinigrin was introduced via the cut petiole into leaves of the non-host plant Vicia faba, the leaves were preferred by B. brassicae over untreated ones. The compound was shown to increase probing and duration of probes into filter papers moistened with sinigrin solution as compared with filter papers moistened with water (Wensler, 1962). When incorporated into a diet containing sucrose, sinigrin rendered the diet more acceptable to B. brassicae (Moon, 1967). In agreement with these results, when a broad range of compounds were tested in artificial diets for effects on M. persicae in dual choice situations, all compounds with the exception of sinigrin (and tomatine) made the diet less acceptable (Schoonhoven and Derksen-Koppers, 1976). Interestingly, it was recently found that sinigrin made artificial diets less acceptable to a Beijing clone of M. persicae (Junde and Lidao, 1984). M. persicae and B. brassicae showed different strategies in dealing with glucosinolates ingested: while M. persicae excreted high concentrations of glucosinolates in the honeydew, B. brassicae retained high concentrations in the body (Weber et al., 1986)

Aphids such as B. brassicae and M. persicae, which have glucosinolate-containing crucifers as hosts, were more stimulated by sinigrin to take up a sucrose-containing diet than R. padi, A. fabae and A. pisum, for which crucifers are non-hosts (Klingauf et al., 1972). Similarly, whereas B. brassicae, M. persicae and Lipaphis erysimi Kaltenbach could be induced to feed on sinigrin-treated non-host leaves, A. fabae, A. pisum and Acyrthosiphon solani could not (Nault and Styer, 1972).

In addition to being phagostimulants, glucosinolates were antibiotics: they decreased survival of L. erysimi in diets (Dilawiri and Atwal, 1987), and positive correlations were obtained between total glucosinolate content of various crucifers and resistance to L. erysimi (Malik, 1981; Gill and Bakhetia, 1985).

4.4. Hydroxamic acids

Hydroxamic acids (Hx) have been shown to play an important role in the interactions of cereals with aphids (Niemeyer, 1988b). Inverse correlations were obtained between Hx levels in maize and infestation by R. maidis, under field and greenhouse conditions (Long et al., 1977; Beck et al., 1983) and between Hx levels in wheat and infestation numbers of Metopolophium dirhodum Walker (Argandoña et al., 1980), S. graminum (Argandoña et al., 1981) and S. avenae (Bohidar et al., 1986) determined in the laboratory.

When DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one), the main Hx in wheat and maize extracts, was infiltrated into barley leaves lacking Hx, the levels of DIMBOA in the leaves correlated inversely with infestation numbers of M. dirhodum (Argandoña et al., 1980)

Hx levels decreased with the age of the plant and the age of the tissue within a plant. Older plants and older leaves of wheat and maize were more heavily infested than younger ones (Argandoña et al., 1980, 1981).

When added to artificial diets, DIMBOA exhibited antibiotic (Zúñiga et al., 1983; Argandoña et al, 1980, 1981, 1983; Long et al., 1977; Corcuera et al., 1985) and antifeedant (Argandoña et al., 1983; Corcuera et al., 1985) effects towards various cereal aphids, the most sensitive being S. graminum and the least sensitive R. maidis. The 2-O-β-D-glucoside of DIMBOA, present in the uninjured plants, was less active than DIMBOA itself (Corcuera et al., 1985).

The toxicity of DIMBOA has been related to its inhibitory action over the mitochondrial energy transduction system (Niemeyer et al., 1986) and, more generally, to its capacity to inhibit various enzymes due to its reaction with nucleophiles such as cysteine and lysine residues (Pjérez and Niemeyer, 1985, 1989a, 1989b).

Higher levels of hydroxamic acids were found in the lateral veins than in the central vein of maize leaves (Argandoña and Corcuera, 1985). In wheat seedlings, the highest levels in the leaf were found in the vascular bundles (Argandoña et al., 1987).

Hydroxamic acids were not detected in the lower epidermis (Argandoña et al., 1987).

DIMBOA was present in aphids which had been feeding on DIMBOA-containing wheat plants (Niemeyer et al., 1989). Aphids feeding on plants with higher DIMBOA levels contained less DIMBOA, produced less honeydew and suffered less weight increase than those feeding on plants with lower DIMBOA levels, indicating a feeding deterrent effect of DIMBOA in the plant. Furthermore, it was found that aphid feeding provoked an increase in Hx levels in some wheat cultivars (Niemeyer et al., 1988). These results open the possibility of diminishing aphid-provoked viral infections in wheat. Screening of ancient and modern wheat germplasm has shown that potentially suitable material is available for breeding high Hx levels into agronomically relevant wheat (Niemeyer, 1988a; Thackray et al., 1989; Copaja and Niemeyer, unpublished results). A further advantage of Hx as a source of resistance in cereals is that its wide range toxicity might provide resistance against a large variety of organisms.

4.5. Other compounds

Benzyl alcohol was claimed to be involved in aphid resistance of barley (Juneja et al., 1972, 1975). However, the presence of benzyl alcohol could not be confirmed in a series of barley cultivars (Zúñiga et al., 1985).

Acceptability to *Aphis craccivora* Koch of V. faba over *Termis lupinus* was associated with lower coumarin levels in the former. Systemic and spraying treatments of V. faba with coumarin diminished the performance of the aphid (Mansour et al., 1983).

Cardiac glycosides are important in the association of the aposematic aphid *Aphis nerii* Boyer with asclepiadaceous plants. Plant-derived cardiac glycosides isolated from the aphid (Rothschild et al., 1970) were ingested by feeding preferentially from the internal primary phloem (Botha et al., 1977).

Numerous compounds of natural origin have been tested in artificial diets for effects on feeding preference and performance of aphids. The compounds tested have included flavonoids and phenolics (Todd et al., 1971; Dreyer and Jones, 1981; Jones and Klocke, 1987), terpenoids (Rose et al., 1981), coumarins (Dreyer et al., 1987), alkaloids (Dreyer et al., 1985 and a variety of other compounds (Herger, 1975; Schoonhoven and Derksen-Koppers, 1976; Dreyer et al., 1981; Campbell and Binder, 1984; Kubo and Klocke, 1983; Kubo et al., 1984).

In most cases, the ecological relevance of the results was not demonstrated, i.e., whether aphids encounter the compounds under natural conditions and whether effects in Nature parallel those obtained with artificial systems. Cyclitols from alfalfa were found in the honeydew of aphids fed with alfalfa (Campbell and Binder, 1984). Pinitol, the most abundant cyclitol in alfalfa showed, however, no effect on A. pisum when added to artificial diets (Campbell and Binder, 1984).

The feeding preferences of aphids is further complicated by variations that may be found among different clones (Weber, 1985 a,b,c), and by the occurrence of different biotypes (Wightman and Gibson, 1972; Eastop, 1973; Campbell et al., 1982; Montllor et al., 1983; Diehl and Bush, 1984; Srivastava et al., 1984; Ryan et al., 1987; Niassy et al., 1987)

5. CONCLUSIONS

The importance of plant volatiles in host selection by aphids is beginning to be recognized. Sophisticated instrumentation available for the observation of insect behaviour and for the analysis of minute amounts of volatiles in complex mixtures, make it possible to increase research efforts in this field. Similarly, the nature of plant surface chemistry should be further studied. Results in these two fields may be particularly beneficial in terms of possibilities of control of virus transmission by aphids.

In the area of internal plant constituents, efforts should be devoted to defining the precise location of secondary compounds in the plant, the chemical nature of phloem sap, the nature and quantity of materials ingested by aphids, and their fate in the aphid.

Secondary metabolites represent an interesting source of resistance in crop plants against aphids. Efforts should be directed towards understanding their mode of action on the recipient organism as well as their biosynthesis. This could lead to a more rational approach towards crop improvement, potentially allowing the obtainment of more stable resistance and the use of genetic engineering techniques. The effect of an increase in levels of secondary metabolites in a crop plant on the

environment as well as on other potential consumers of the plant should also be considered.

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29

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Resistance to the aphids *Sitobion avenae* and *Rhopalosiphum padi* in Gramineae in relation to hydroxamic acid levels

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Summary

Antibiotic resistance to the aphid *Sitobion avenae* was assessed in relation to levels of hydroxamic acids (Hx) in a wide genetic range of cultivars and species of *Triticum*. Within hexaploid and tetraploid *Triticum* material, total plant concentrations of Hx explained a significant proportion of the variation in intrinsic rate of increase (r_m) of *S. avenae*. Significant correlations were also found between resistance to *Rhopalosiphum padi* and Hx levels. Although the concentrations of Hx in whole plants declined during seedling growth, concentrations of Hx in newly-emerging leaves remained high in plants of all ages, including in the emerging flag leaves of mature plants. When the mean relative growth rate of *S. avenae* over three days was used instead of r_m and the control of environmental conditions was improved, a higher proportion of the variation in aphid performance was explained by Hx concentrations in six cultivars.

Key words: Resistance, Gramineae, aphids, hydroxamic acids

Introduction

Partial host-plant resistance can make a substantial contribution to reducing the damaging effects of cereal aphids and other pests and therefore to reducing insecticide use. However, aphid resistance has not been deliberately bred into any UK wheat variety (Vickerman & Wratten, 1979). The possibility of exploiting inherited, low-level resistance in wheat has prompted the screening of many British cultivars and breeding lines of hexaploid wheat, *Triticum aestivum* L., but resistance levels have not varied markedly (Lowe, 1981, 1982). An exception has been the demonstration of the role of awns in wheat in conferring aphid resistance (Acreman, 1984), although this has also not been exploited by plant breeders. Recently interest has moved towards wheat species other than *T. aestivum*, notably the diploid Einkorn wheat, *Triticum monococcum* L., an ancestor of *T. aestivum*, which shows resistance to the aphids *Metopolophium dirhodum* Wlk. and *Sitobion avenae* F. (Spiller & Llewellyn, 1986; Sotherton & Lee, 1988) and the tetraploid *Triticum durum* Desf., cultivars of which show resistance

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30

to the aphid *S. avenae* (Bohidar, Wratten & Niemeyer, 1986). The progress of plant breeders in the search for resistant genes is however still restrained by the absence of a reliable, rapid and convenient assay for resistance, and by the lack of information on the mechanisms of resistance when it is found and on its genetic basis.

Hydroxamic acids (Hx) occurring in cereal extracts, in particular the compound 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), have been shown to be involved in the resistance of cereals to bacteria (Corcuera *et al.*, 1978), fungi (ElNaghy & Linko, 1962) and several insects including the aphid species *M. dirhodum*, *Schizaphis graminum* Rond. and *Rhopalosiphum maidis* Fitch (Corcuera, Argandoña & Niemeyer, 1982). *S. avenae* has been investigated in this context only in preliminary work by Bohidar *et al.* (1986) who found that 96% of the variance in the resistance of seedlings of six cultivars was explained by concentrations of Hx.

Hx exist in the plant as glucosides and are enzymically hydrolysed to release the active aglucone upon injury (Hofman & Hofmanova, 1969). Antifeedant properties of Hx towards aphids have been shown in diets (Argandoña, Corcuera, Niemeyer & Campbell, 1983) and in plants (Niemeyer, Pesel, Franke & Francke, 1989). Antibiosis of Hx has also been shown in diets (Corcuera, Argandoña & Niemeyer, 1982). Whilst Hx are found only at low levels in whole plant analyses of most modern hexaploid wheats, higher concentrations have been associated with lines of some tetraploid wheats such as *T. durum* cultivars SNA3 and SNA2 (Bohidar *et al.*, 1986). Niemeyer (1988) examined Hx levels in other tetraploid *Triticum* species.

The objectives of the present investigation were 1) to assess antibiotic resistance to the cereal aphids *S. avenae* and *Rhopalosiphum padi* L. in relation to levels of Hx in a wide genetic range of cultivars and species of the genus *Triticum* and 2) to attempt to explain the residual variation by analysis of the temporal and spatial distribution of Hx within the plant. Young plants were used for most of the work although some analyses were carried out on plants at a range of growth stages including plants at the grain development stage. There were two reasons why mainly seedlings were used. Firstly, the concentration of Hx for the total plant is highest at seedling emergence and declines with age in most wheats (so that quantification of lower concentrations becomes increasingly less accurate). Secondly, whilst *S. avenae* can be particularly damaging to mature plants during grain-ripening it and *R. padi* can be important pests of young seedlings in the autumn, as vectors of BYDV (barley yellow dwarf virus).

Materials and Methods

Choice of plant material

The seed material used in this study was chosen using criteria based on the phylogeny of *Triticum aestivum* (Riley, 1965). A total of 20 lines were investigated including representatives of both hexaploid and tetraploid *Triticum* species (Fig. 1). There were seven Chilean cultivars: Naofen, Likay, Sonka, Huenufen, SNA3, SNA2, Quilafen. *Triticum durum* cv. SNA3, *T. aestivum* cvs Avalon and Jerico were used to examine temporal variation of concentrations of Hx. These lines represented high, medium and low concentrations of Hx respectively. *T. durum* cv. SNA3, and *T. aestivum* cv. Likay were used to study the spatial variation in concentrations of Hx in young seedlings. These lines were chosen for their high and low concentrations of Hx respectively. The examination of Hx levels in four different growth stages of mature wheat utilised *T. aestivum* cv. Mission, which was grown on a field site at Chilworth, Hampshire. For investigating the relationship between concentrations of Hx and the mean relative growth rate of *Sitobion avenae* on a small group of taxa grown under carefully controlled conditions, six lines were selected: *T. aestivum* cvs Avalon, Armada, Apostle, Naofen, Likay and *T. durum* cv. SNA3. They were again chosen to represent a range of hydroxamic acid concentrations and were also readily available in sufficient quantities of seed.

Assessment of the relationship between the intrinsic rate of increase of aphids and concentrations of hydroxamic acids

The methods used for producing test insects and plants for assessment of Hx concentration and aphid performance were similar to those used by Bohidar *et al.* (1986), with the exception that fecundity was recorded over five days only (when nymph production was highest), rather than over 10 days; the shorter period was found to be sufficient to give consistent and reliable differences in the values for the intrinsic rate of natural increase (r_m; Birch, 1948). The r_m values for *Sitobion avenae* on each cultivar were calculated using a computer program incorporating the 'Jack-knife' technique to give standard errors (Miller, 1974; Bissel, 1977; Birch & Wratten, 1984). Aphids were introduced onto the plants at the early two-leaf stage (G. S. 11-12, Zadoks, Chang & Konzak, 1974), about 7 days after seedling emergence; at the same time, concentrations of Hx were determined in uninfested plants of the same cohort.

The method of Hx extraction followed that of Bohidar *et al.* (1986) and was based on the colorimetric absorption of a hydroxamic acid-ferric chloride complex. This procedure does not differentiate between the different hydroxamates present in extracts; however, Niemeyer *et al.* (1989*b*) using high performance liquid chromatography (HPLC) found that DIMBOA was the main or only Hx in the Chilean wheats. Zuñiga, Argandoña, Niemeyer & Corcuera (1983) determined concentrations of Hx in a wide variety of wild and cultivated Gramineae using both the ferric chloride method and a TLC-UV procedure. The latter technique differentiates between individual hydroxamic acids. The total Hx concentration determined by the ferric chloride method was consistently slightly higher than that determined by the TLC-UV method (on average 10% higher) and the authors suggested that this might be due to the presence of other hydroxamic acids in the extracts. There is a potential danger that ferric chloride will react with phenolic substances present in the extract since it is not a specific reagent for Hx. Extracting samples into ether attempts to avoid this problem. However, in those species where no DIMBOA or DIBOA were detected by TLC-UV methods, the ferric chloride procedure also failed to detect any reactive substances, suggesting that in this *Triticum* system there is no problem arising from the non-specificity of the reagent. A comparison of ferric chloride and GLC methods was made in maize by Woodward *et al.* (1979) which showed a direct relationship between DIMBOA determined by GLC and total hydroxamates determined by the ferric chloride method.

The relationship between r_m of *S. avenae* and concentrations of Hx in 7-day-old seedlings for each variety was tested using regression analysis. Similar analyses were performed on the data for *Rhopalosiphum padi* feeding on a selected group of plant taxa.

Temporal and spatial variation in hydroxamic acid concentrations

A study of temporal and spatial changes in concentrations of Hx in selected taxa was made. Hx were assessed in whole plants of a variety of ages and individual assessments were made of leaves of different ages in two-, three-, four- and five-leaved plants.

For the examination of the temporal variation of concentrations of Hx, seeds of each variety were sown on the same day and grown under identical conditions to those used in Bohidar *et al.* (1986). Within each cohort, seedlings were harvested at daily intervals from two days after germination up to the eleventh day. Hx are absent in the seed itself (Argandoña, Niemeyer & Corcuera, 1981). Four 1 g samples of leaf and stem material (including the leaf sheath) of each cultivar were required each day for establishing the concentrations of Hx in whole plants. Differences in temporal variation between lines were analysed using a two-way analysis of variance followed by Tukey's multiple range test, having established the comparisons to be made before the test was carried out (Sokal & Rolf, 1981). In a subsidiary experiment

32

flag leaves were collected from field-grown *T. aestivum* plants (cv. Mission) of known growth stages. Four 1 g samples taken from a mixture of dissected flag leaves from different plants were used in the analysis of flag leaves at each growth stage.

In order to examine variation in concentrations of Hx between the different leaves of young seedlings, similar procedures were used. A proportion of the seedlings within each cohort were harvested as each new leaf emerged, at the two-, three-, four- and five-leaf stages, enabling leaf blade samples at each growth stage to be analysed for temporal variation in concentrations.

Assessment of the relationship between aphid mean relative growth rate and hydroxamic acid levels in six cultivars

Since the measurement of aphid performance used in the major screening of 20 lines was made over a considerable period of time (at least 14 days), fluctuations in Hx levels could have contributed to the variation found in the relationship between r_m and concentrations of Hx. Variation may also have been attributable to small changes in environmental conditions over the period of assessment. Leather & Dixon (1981) showed a strong correlation between r_m and the mean relative growth rate (mrgr) of *R. padi*. For the re-assessment of six lines under carefully controlled environmental conditions, mrgr was used so as to considerably shorten the length of the assessment period required to give a reliable measure of aphid performance on each line.

The seeds of all six cultivars were germinated on damp filter paper in complete darkness at 15 ± 1°C. After four days, groups of six seedlings were planted in 9-cm diameter plastic pots containing John Innes No. 2 seed compost, given a drench of the fungicide ethyrimol (as Milstem) to limit mildew infection and kept on trays in culture rooms as described in Bohidar *et al.* (1986). Transparent plastic tubes 8.5 cm in diameter and 24 cm high with Terylene mesh tops, were then placed over each pot so as to mimic the conditions described below for the mrgr experiments. These pots were arranged in a six by six Latin square arrangement and the seedlings harvested for Hx analysis seven days after germination, at growth stage 11-12, separated into individual leaves, weighed into four 1 g samples and stored at −20°C for later analysis.

For examining aphid performance on the different cultivars, seeds were germinated as above and 25 seedlings of each variety planted individually in 7-cm diameter plastic pots as above. Seven days after germination, one previously weighed second-instar aphid was gently introduced onto the oldest leaf of each seedling and a transparent plastic tube 6.5 cm in diameter with a Terylene mesh top placed over the plant to restrict aphid movement. The pots were positioned randomly on trays in culture rooms as described in Bohidar *et al.* (1986). All pots were watered with Hewitt's Long Ashton Solution (Hewitt, 1966) every two days to prevent depletion in essential nutrients or water supply.

The methods for culturing and selecting aphids were as above. Aphids were monitored daily, with their position on the plant noted, and then re-weighed three days (72 hours) after being placed on the plant. The mean relative growth rate for each aphid species on each cultivar was calculated following the method of van Emden (1969) as:

$$\frac{\log_e \text{final weight} - \log_e \text{initial weight}}{3}$$

One-way analysis of variance was used to test for differences in Hx concentrations between different pots. The relationship between mrgr and concentrations of Hx in 7-day old seedlings of each variety was tested using regression analysis.

Results

The relationship between concentrations of Hx and r_m of *Sitobion avenae* for hexaploid and tetraploid *Triticum* material is shown in Fig. 1. Values for concentrations of Hx ranged from 1.98 to 27.15 m mole/kg dry weight and were particularly high in some of the tetraploid wheats such as *Triticum durum* cv. SNA3, *Triticum turgidum* L. and *Triticum dicoccum* (Shrank.) Schulb. Values for r_m ranged from 0.140 to 0.309, the highest values (indicating the least resistance) being associated predominantly with the hexaploid wheats and the lowest values with some of the tetraploid wheats. The total concentration of Hx in the plant explained a significant proportion of the variation in intrinsic rate of increase of *S. avenae* on hexaploid and tetraploid *Triticum* material ($\log y = 0.61 - 0.15 \log x$; $r = -0.59$, $P < 0.01$). For *Rhopalosiphum padi* on hexaploid and tetraploid wheat, the relationship between r_m concentrations of Hx was also significant ($\log y = 0.51 - 0.04 \log x$; $r = -0.89$, $P < 0.05$), but the range of the r_m values obtained was small within the limited group of material studied.

The temporal variation in concentrations of Hx seen in three cultivars is shown in Fig. 2. Although total plant concentrations of Hx declined rapidly during the early seedling growth period, they did not fall to zero and the results from Tukey's multiple range test showed that the rate of decline varied significantly between each line. In all three lines studied the rate of decline was greatest during the first 4 days following germination, after which levels declined very slowly or not at all (Fig. 2).

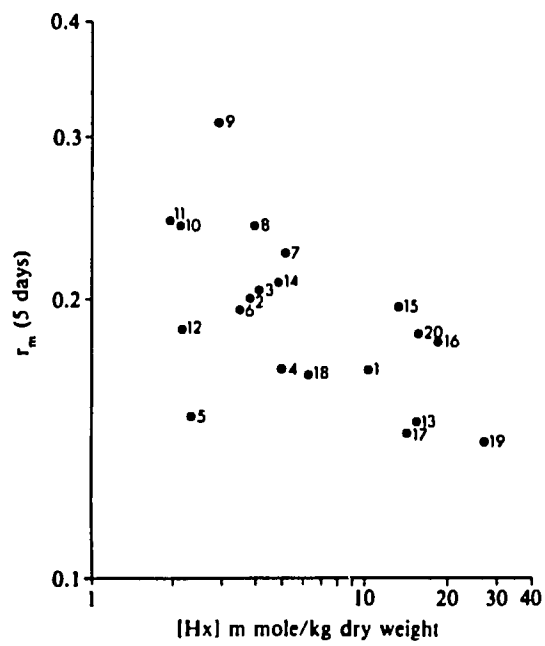

Fig. 1 The relationship between intrinsic rate of natural increase (r_m) of *Sitobion avenae* and levels of total hydroxamic acids [Hx] in 7-day-old seedlings of hexaploid and tetraploid *Triticum* ($\log y = 0.61 - 0.15 \log x$; $r = -0.59$; $P < 0.01$). Numbers denote lines. The species are: 1-12 = *Triticum aestivum*, 13-16 = *Triticum durum*, 17-18 = *Triticum polonicum*, 19 = *Triticum turgidum*, 20 = *Triticum dicoccum*. (1) Avalon, (2) Armada, (3) Hobbit, (4) Musket, (5) Jerico, (6) Nacfen, (7) Likay, (8) Sonka, (9) Huenufen, (10) Bezostaya, (11) Cappelle, (12) 15189, (13) SNA3, (14) SNA2, (15) Quilafen, (16) 435016, (17) 384345, (18) 384338, (19) 352542, (20) 319869.

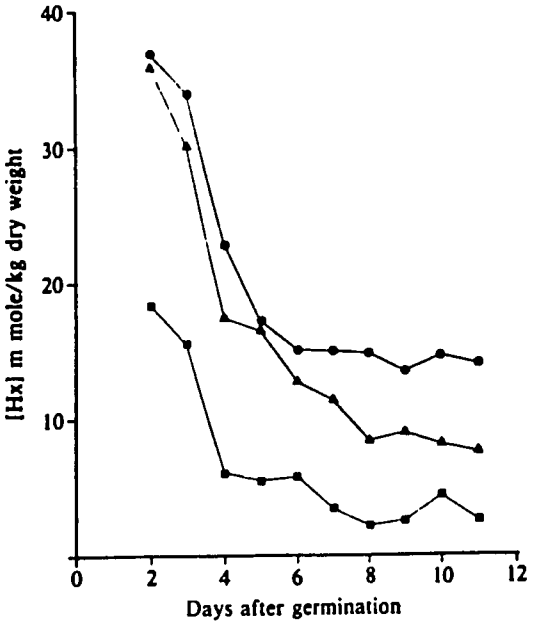

Fig. 2 Variation of concentrations of hydroxamic acids in whole plants in relation to age in three seedling cultivars. Key: ● *Triticum durum* cv. SNA3; ▲ *Triticum aestivum* cv. Avalon; ■ *Triticum aestivum* cv. Jerico.

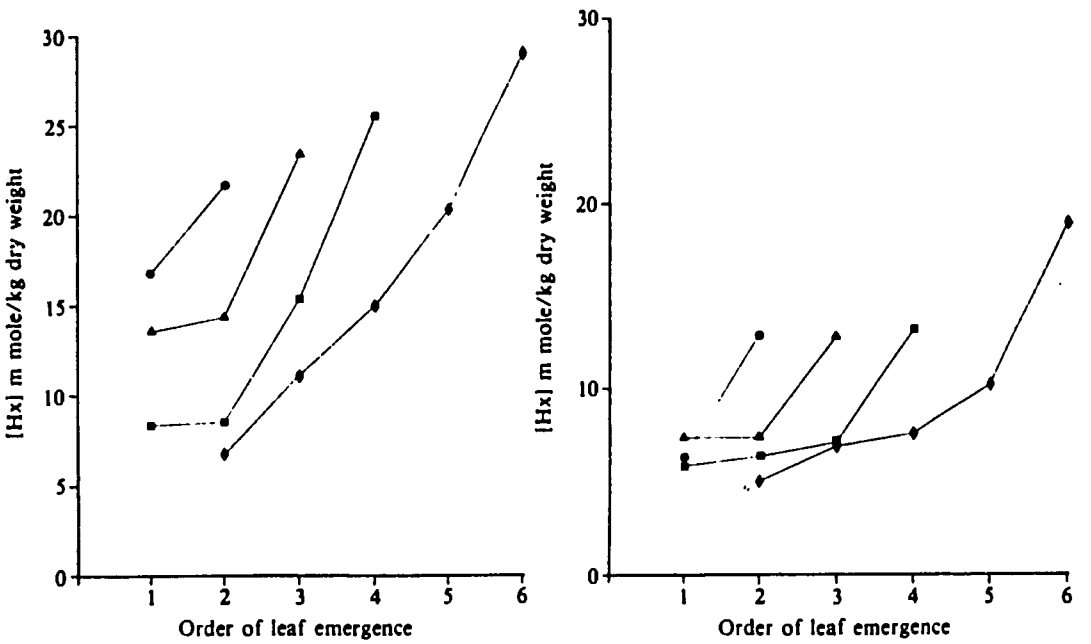

Fig. 3 The concentration of hydroxamic acids [Hx] in individual leaves of a. *Triticum durum* cv. SNA3 and b. *Triticum aestivum* cv. Likay, of plants at the ● 2-, ▲ 3-, ■ 4- and ◆ 6-leaved stages.

Concentrations of Hx were highest in the emerging leaves of seedlings of *T. durum* cv. SNA 3 and *T. aestivum* cv. Likay but declined sharply as the leaves aged (Fig. 3). At all seedling ages, the last leaf to emerge had the highest concentrations of Hx. Concentrations of Hx in the maturing leaves of a four-leaved plant of SNA3 ranged from 8.4 to 15.3 m mole/kg dry weight but a much higher concentration of 25.5 m mole/kg dry weight was found in the

Table 1. *Hydroxamic acid concentration in the flag leaves of mature plants of* Triticum aestivum *cv. Mission*

Crop stage	[Hx] m mole/kg dry weight	95% confidence limit lower	upper
Half inflorescence emerged	10.8	10.4	11.2
Emergence complete	9.2	8.9	9.6
Antithesis complete	5.1	4.7	5.4
Early milk	1.1	0.8	1.5

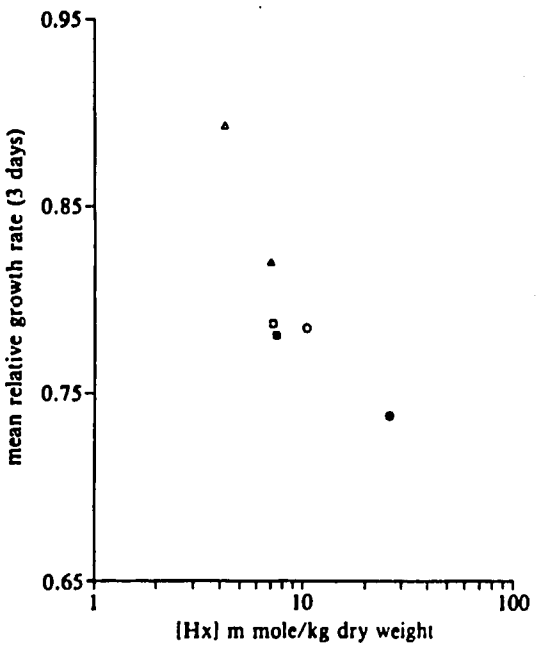

Fig. 4 The relationship between mean relative growth rate of *Sitobion avenae* and hydroxamic acid levels [Hx] in the first leaf of 7-day-old seedlings of six cultivars. Key: ● SNA3, ■ Naofen ▲ Likay, ○ Avalon, □ Apostle, △ Armada.

emerging fourth leaf. Similarly in mature plants of *Triticum aestivum* cv. Mission, the newly emerging flag leaf had relatively high concentrations of Hx (10.8 m mole/kg dry weight), which declined rapidly during anthesis to 1.1 m mole/kg dry weight (Table 1).

When mean relative growth rate was used as a measure of performance for *S. avenae* on six selected cultivars under controlled environmental conditions, the correlation between aphid performance and Hx levels in the oldest leaf was very strong ($r = -0.69$, $P < 0.05$) (Fig. 4).

36

Discussion

Although resistance to aphids in modern wheat is generally low, a wide range of resistance can be seen when the range of genotypes screened is increased. Hydroxamic acids (Hx) explained 35% of the resistance to *Sitobion avenae* in a selection of 20 seedling tetraploid and hexaploid wheats when r_m was the measure of aphid performance. When environmental conditions were more strictly controlled and mean relative growth rate over three days used, 47% of the resistance to *S. avenae* was explained by Hx in the oldest leaf of six seedling wheats. It is unlikely that any single plant characteristic could account for all variation in plant resistance; however, with a greater understanding of the behaviour of Hx in cereals and their effects on aphid feeding behaviour it may be possible to explain some of the residual variation in the relationship between concentrations of Hx and r_m.

It is possible that the effect of fluctuating environmental conditions such as water availability and light intensity could have contributed to the high variation found in the relationship between r_m and concentrations of Hx. Manuwoto & Scriber (1985) found that low-intensity light produced higher levels of Hx in maize plants than in controls. Hx levels are also affected by photoperiod (Epstein, Rowsemitt, Berger & Negus, 1986). In the first major screening of 20 cultivars in the present work, not all lines could be grown and assessed on the same date due to limitations on space and time. Consequently there may have been small variations in the environmental conditions under which different cultivars were grown. When the period of assessment of aphid performance was reduced from around 13-18 days (for r_m assessments) to three days (for mrgr assessments) and the plant growing conditions kept constant by careful attention to water and nutrient supply for a group of cultivars all grown over the same time period, a stronger relationship was established between aphid performance and levels of Hx (47%).

It remains to be established whether and, if so how *S. avenae* on wheat ingests Hx and at what concentrations in the plant they become an important resistance mechanism. This study has shown that concentrations of Hx vary between taxa and are always highest in newly emerging leaves. Thus, the correlation between the aphids' feeding site on the plant and the local concentrations of Hx may greatly affect the probing and settling behaviour of the aphids, given that antifeedant properties of Hx have been demonstrated. Inverse relationships between DIMBOA levels in aphids feeding on wheat plants and DIMBOA levels of the plants themselves have been found (Niemeyer *et al.*, 1989*b*), indicating possible feeding deterrency by DIMBOA. In addition, the relationship between aphid performance and concentrations of Hx should be re-examined in the light of recent evidence that localised aphid damage to wheat can induce increases in concentrations of Hx (Niemeyer *et al.*, 1989) and also in aphid nymphal mortality (Thackray, Morse & Leech, 1988). Inducibility of Hx in different cultivars may vary greatly.

It was previously thought (Argandoña *et al.*, 1981) that concentrations of Hx decline rapidly during seedling growth, reaching very low levels in maturing plants; however, the present study has shown that concentrations may be relatively high in newly emerging leaves, including emerging flag leaves. For example, concentrations of Hx in the newly emerged flag leaves of *Triticum aestivum* cv. Mission were 10.8 m mole/kg dry weight, compared with values of 12.9 and 21.7 in the new leaves of 9-day old seedlings of *T. aestivum* cv. Likay and *Triticum durum* cv. SNA3 respectively. In a tillering plant, a fairly high proportion of leaf material might therefore be high in concentrations of Hx, thus giving a greater degree of protection to the young tissues of the plant than was previously supposed. The localisation of Hx in areas of sensitive new growth has important implications for the plant's resistance to aphid damage. Until recently Hx were not thought to be of importance in the resistance of mature wheat plants to insects, but the evidence now suggests that they may play a role in wheat plants of all ages against aphid attack. Leszczynski, Wright & Bakowski (1989), for instance, demonstrated a highly significant negative correlation ($r = 0.905$) between *S. avenae* r_m and Hx for flag leaves.

Further studies of aphid behaviour on resistant and susceptible cultivars, incorporating a range of concentrations of Hx, are required together with studies of the relationship between concentrations of Hx and survival of other cereal pests. A negative relationship between concentrations of Hx and r_m for *Rhopaiosiphum padi* has already been shown in the present study and future investigations will consider this and other cereal aphids with Hx levels determined in particular plant parts in relation to feeding behaviour, such as the leaf sheaths where *R. padi* mainly feeds.

From this study, a useful matrix of data relating to the taxonomic pattern of resistance within *Triticum* has emerged and it is hoped that the increasing knowledge of the behaviour of Hx within the plant might lead to the use of hydroxamic acid analysis in plant breeding programmes, both as a standard when selecting promising lines for resistance to cereal aphids and also in the isolation of resistant genes.

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34

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

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(*Received in revised form* 9 *August* 1989)

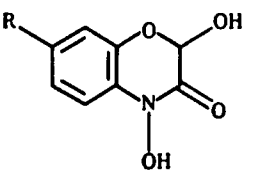
Key Word Index—*Avena fatua*; *A. sativa*; Gramineae; allelopathy; hydroxamic acids; benzoxazinones.

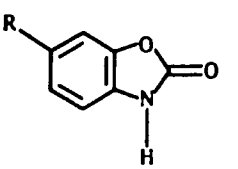
Abstract—2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), the main hydroxamic acid of wheat, and its decomposition product 6-methoxy-benzoxazolin-2-one (MBOA), inhibited 50% root growth of wild oat, *Avena fatua* at concentrations of 0.7 and 0.5 mM respectively. 6-Methoxy-benzoxazolin-2-one also inhibited seed germination of *A. fatua* at all concentration tested. It stimulated root growth in *A. sativa* at concentrations below *ca* 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 hr. Uptake by *A. fatua* seeds of MBOA, DIMBOA and water showed similar kinetic patterns. However MBOA was taken up preferentially to DIMBOA. The MBOA uptake depends on the after-ripening of the seed. The potential exploitation of hydroxamic acids from wheat as allelochemicals in the control of *A. fatua* is discussed.

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 post-emergence 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) **1–4** 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* and *A. sativa* (a crop normally rotated with it).





	R
1	OMe
3	H
2	OMe
4	H

RESULTS

Effect on root length

2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one inhibited root growth of *A. fatua* (I_{50} = 0.7 mM), but the inhibitory effect was significantly smaller in *A. sativa*. Although MBOA also inhibited the root growth of *A. fatua* (I_{50} = 0.5 mM), 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 hr at pH 5 [13]. Because bioassays were carried out in distilled water pH 5, the effects exhibited by DIMBOA may not be due to the compound itself. Quantitation of DIMBOA and MBOA in *A. fatua* seeds was carried out. *Avena fatua* seeds were submitted to a pulse of DIMBOA (4 mM, 4 hr) and then transferred to a medium containing only water. The 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 hr.

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. By contrast, all concentrations of MBOA inhibited germination with completeness being reached at 4 mM (Fig. 2).

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

Incubation with 4 mM DIMBOA (hr)	Inhibition in water (hr)	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 ± 1.2

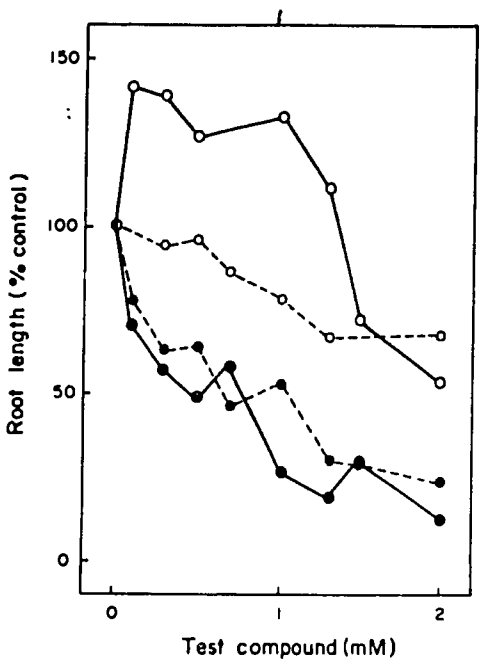


Fig. 1. Inhibition and stimulation of root growth of wild oat *A. fatua* (●) and oat *A. sativa* (O) by MBOA (——) and DIMBOA (---) after three days incubation in the dark at 20 + 3°. LSD relative to control for *A. fatua*: DIMBOA (5–5%) and MBOA (5–9%). LSD relative to control for *A. sativa*: DIMBOA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

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 after-ripening 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 non-dormant *A. fatua* seeds was also measured (Fig. 4). Significant differences were found.

DISCUSSION

Release and uptake of hydroxamic acids

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

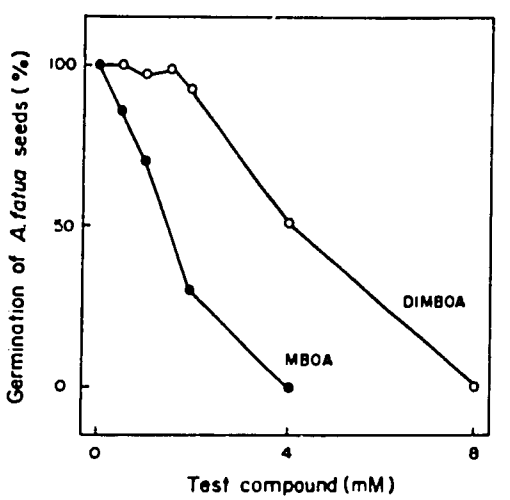


Fig. 2. Inhibition of germination of *A. fatua* by MBOA (●) and by DIMBOA (O) after six days incubation in the dark at 20 + 3°.

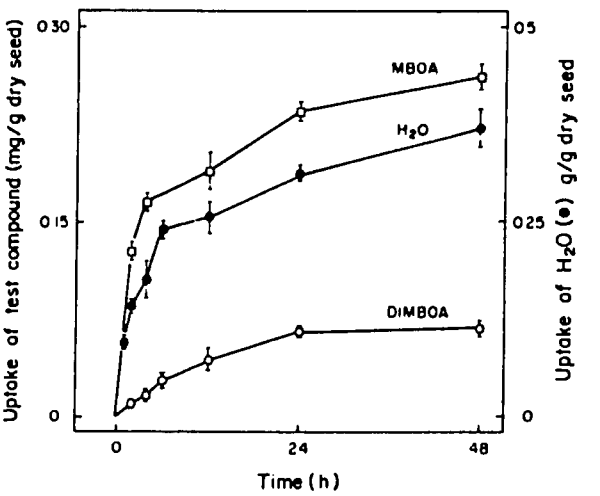


Fig. 3. Kinetics of uptake of DIMBOA (O), MBOA (□) and water (●) by dormant *A. fatua* seeds. The concentration of test compounds was 4 mM.

41

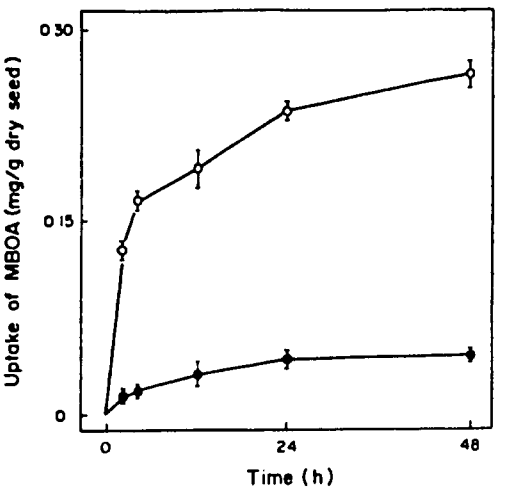


Fig. 4. Uptake of MBOA by dormant (O) and non-dormant (●) *A. fatua* seeds.

decomposition product BOA, are phytotoxic towards several monocotyledonous and dicotyledonous 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 that act as allelochemicals are the corresponding benzoxazolinones.

Significant differences in MBOA uptake were found between dormant and non-dormant *A. fatua* seeds (Fig. 4). For example, after 48 hr of imbibition in 0.5 mM MBOA (corresponding to I_{50}) 0.23 μg of MBOA were found inside each non-dormant seed. In dormant seeds, this quantity of MBOA would be expected to be reached with an MBOA concentration in the imbibition medium sixfold 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 after-ripening period to which the seed being tested has been subjected. The change in MBOA uptake with the length of the after-ripening 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 yet known. Two alternatives have been reported to account for their phytotoxic effects. Both DIMBOA and MBOA modify the binding affinity of 1-naphthylacetic 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 inhibits 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 to interference with the normal activity of auxin. Further studies are underway to test this hypothesis.

Potential uses of hydroxamic acids as allelochemicals

Strategies for utilizing allelopathy as an aid in crop production are receiving much attention [7-9]. In this paper, it is shown that Hx isolated from wheat inhibited root growth and seed germination of *A. fatua*, an annual grass weed associated to this crop. It is 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 *Triticum durum* cv. SNA-3, grown in a greenhouse at 25 ± 3°. MBOA was synthesized as described in ref. [20].

Seeds. Seeds of *Avena fatua* were obtained from the Agricultural Experimental Station Quilamapu (INIA), and were collected in 1986 and stored under dry conditions for 2 years. These seeds were non-dormant and had a germination rate higher than 95%. Dormant *A. fatua* seeds that had not completed their after-ripening 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 No 1 filter paper, and 2.5 ml H_2O were added. The dishes were incubated in the darkness at 20 ± 3°. Seeds were considered germinated when the radicle appeared through the testa.

Bioassays. Bioassays were carried out in Petri dishes, each containing one disk of Whatman No 1 filter paper. Appropriate solutions of DIMBOA and MBOA in Me_2CO were added to the filter paper. Pure Me_2CO, similarly applied to the filter paper was used as control. After solvent evapn 2.5 ml of dist. H_2O 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 triplicate and the *t*-test was applied for significant differences.

Kinetics of uptake of DIMBOA, MBOA and H_2O *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 a 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 14000 *g* 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 with the HPLC analysis. After this treatment, the methanolic extract was evapd 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 C 18 Micro Pak MCH-10 column (300 × 4 mm) and UV detector (at 254 nm) or in a Shimadzu LC-6A liquid chromatograph, equipped with a Lichrocart C18 column (125 × 4 mm) and a variable UV/Vis detector (at 290 nm). The

mobile phase consisted of a mixture of 70% acidic H_2O (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 analysed 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 hr. After this time, the seeds were washed twice with H_2O, 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.

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43

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

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(*Received in revised form* 13 *October* 1989)

Key Word Index— Gramineae; benzoxazinones; hydroxamic acids; DIMBOA; α-chymotrypsin plant defence.

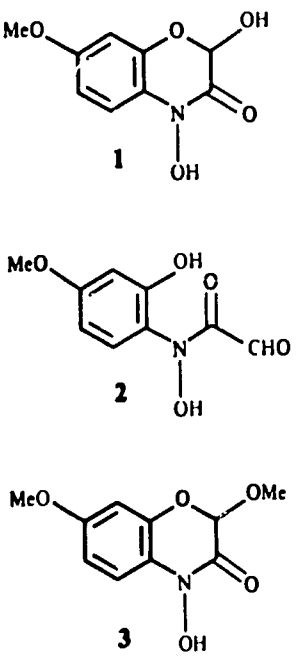
Abstract- -2,4-Dihydroxy-7-methoxy-1, 4-benzoxazin-3-one (DIMBOA), a hydroxamic acid involved in pest resistance of cereals, inactivated α-chymotrypsin (EC 4.21.2). Semilog plots of the residual activity of α-chymotrypsin in the presence of an excess of DIMBOA as a function of time were not linear. A 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. Amino acid 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 implicated as a resistance factor toward the European corn borer, *Ostrinia nubilalis*, a major pest of corn [2]. When larvae of *O. nubilalis* were 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 the 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 both *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.

Because 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 DIMBOA inhibition of *O. nubilalis* proteinases.



RESULTS AND DISCUSSION

Kinetic analysis

Semilog plots of the decrease with time of α-chymotrypsin activity in the presence of an excess of DIMBOA at pH 7.5 were not linear. After 8 hr, complete inactivation was not obtained at any 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 hr only

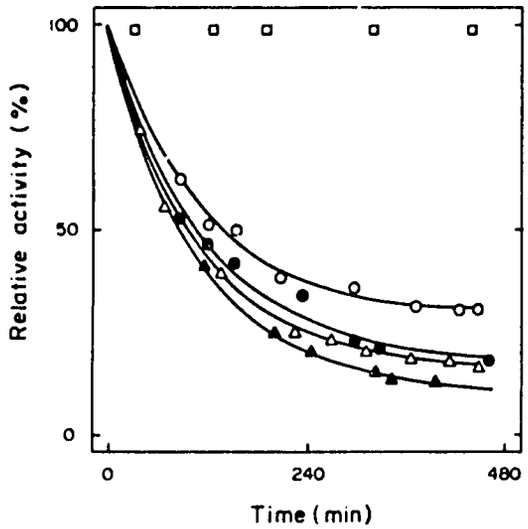
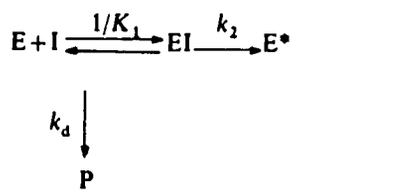


Fig. 1. Kinetics of α-chymotrypsin inactivation by DIMBOA at different concentrations: 0 (□); 10 (○); 15 (●); 20 (△) and 40 (▲) mM. The curves represent the least-squares fit of the experimental points to equation 1. Initial specific activity was 4.9 μmol/min mg protein.

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.



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

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 eqn 1 was transformed to eqn 3 by setting $t = \infty$ and establishing the condition $(I)_o \gg K_1$.

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

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

The experimental points for the time dependence inactivation of α-chymotrypsin at different DIMBOA concentrations were least-squares fitted to eqn 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 (eqn 3) did not differ significantly from those calculated above (Table 1).

Table 1. k_2 and K_1 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° ($k_d = 8 \times 10^{-2}$ min^{-1}, [4])

DIMBOA (mM)	k_2	$K_1 \times 10^{-3}$
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_2 and K_1 values determined through the end point method (equation 3) were 0.076 ± 0.0063 min^{-1} and 5.2 ± 1.3 × 10^{-2} M, respectively.

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. Amino acid 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 amino acids 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 serin in α-chymotrypsin [14] warranted the parallel determination of the 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 precision of amino acid 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 its aldol (2) with which it forms an equilibrium mixture in solution [16]. Hence, inactivation experiments were carried out with 4-hydroxy-2,7-dimethoxy-1,4-benzoxazinone (3), a compound that cannot 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,β-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]. It appears likely that the toxicity of DIMBOA towards phytophagous insects such as *O. nubilalis* is

45

Table 2. Relative activities of α-chymotrypsin inactivated by 30 mM DIMBOA after incubation (pH 7.5, 30°) for 3 hr 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.

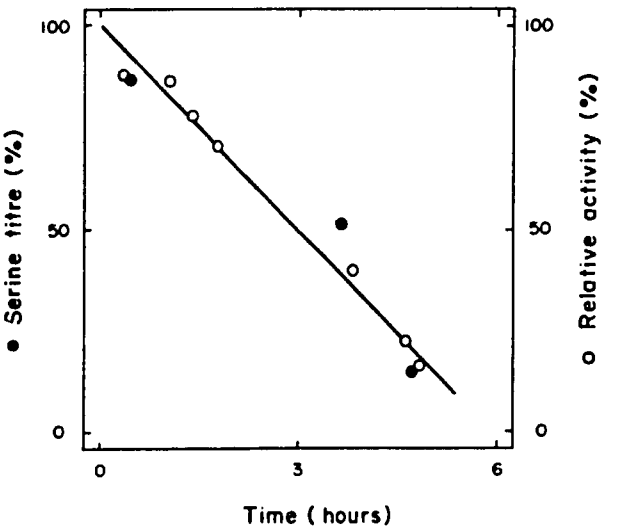


Fig. 2. Proportionality of α-chymotrypsin activity and serine titre as percentage of initial values, during the inactivation of the enzyme by DIMBOA. Initial specific activity was 4.9 μmol/min mg protein and initial serine titre was 0.97 mol serine/mol enzyme.

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

Amino acid	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 mol protein. Data was normalized with respect to arginine and threonine.

†Not detected by this method.

related to its capacity to inhibit serine or thiol [18] proteinases in the larval digestive tract.

EXPERIMENTAL

Enzymes and reagents. α-Chymotrypsin (3 × 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 (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 in ref. [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 A_{400} due to the release of p-nitrophenol was followed [20]. The reaction mixture consisted of 14 μl 3.2×10^{-4} M substrate, 2.6 ml 0.1 M MOPS buffer with 5 mM $CaCl_2$, pH 7.5 (buffer A), 350 μl MeCN and 5 μl 3.2×10^{-6} M α-chymotrypsin. The non-enzymatic hydrolysis of the substrate was taken into account by adding the substrate to the reference cuvette.

Inactivation of α-chymotrypsin by DIMBOA. The reactions were followed under pseudo-first order conditions with an excess of 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 soln (14 μl) of 3.2×10^{-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 1.6×10^{-4} M α-chymotrypsin and 40 mM DIMBOA was incubated in buffer A at 30° for 12 hr. 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 concd by ultrafiltration and then lyophilized. The protein was hydrolysed with 6 M HCl at 110° for 24 hr and analysed in a Beckman amino acid analyzer model 120. 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 in ref. [15]. To a mixture containing 2.9 ml 0.1 M acetate buffer with 3.2% of MeCN, pH 5, and 24 μl 10 mM N-*trans*-cinnamoylimidazol (ε 9115 M^{-1} cm^{-1}), different vols of 3.2×10^{-4} M α-chymotrypsin were added. Decrease in A_{325} was measured with respect to a control without enzyme. The enzyme concentration was measured at 280 nm (ε 50000 M^{-1} cm^{-1}) [21]. A relation of 0.97 mol 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 3.2×10^{-4} M α-chymotrypsin, 1.9 ml buffer A and 100 μl 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.

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47

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ROOT EXUDATES OF WILD OATS: ALLELOPATHIC EFFECT ON SPRING WHEAT

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Key Word Index—*Avena fatua*; *Triticum aestivum*; Gramineae; root exudates; allelopathy; scopoletin; coumarin; *p*-hydroxybenzoic acid; vanillic acid.

Abstract—Root exudates from the undisturbed root system of wild oats *Avena fatua* were collected by a modification of the Tang and Young method. Exudates inhibited root and coleoptile growth of spring wheat seedlings (*Triticum aestivum*). Scopoletin, coumarin, *p*-hydroxybenzoic and vanillic acid were tentatively identified from the root exudates by HPLC.

INTRODUCTION

Wild oats, *Avena fatua* L., is one of the most troublesome annual grass weeds of wheat and other cereals [1]. The persistence and irregular germination of its seeds are major contributing factors to its success [2]. Infestation by wild oats causes extensive yield losses in cereal crops [3–5]. This detrimental effect has been explained by plant competition for light, moisture and nutrients [6]. However, it has recently been suggested that allelopathy could also be involved in the reduction of yield crop by wild oats [7]. The release of allelochemicals by the plant in sufficient quantities to elicit a response in the target plant or seed remains a critical question in studies of allelopathy [8]. One of the main mechanisms by which plants can release allelochemicals to the soil is through root exudation.

In this paper root exudates from undisturbed wild oat roots were collected at different growth stages by a modification of the Tang and Young method [9]. Toxic effects of these exudates were assessed through bioassays on spring wheat (*Triticum aestivum* L.) seedlings. Tentative identification of allelochemicals present in the root exudates were performed by comparing their R_ts times on HPLC with those of standard compounds.

RESULTS

Bioassays

Root exudates collected between the emergence and the first leaf stage of wild oat inhibited root and coleoptile growth of spring wheat. In both cases (Figs 1 and 2) inhibition increased with the volume of root exudate tested. As the amount of solids employed in bioassays was very low (0.2–2.5 mg) the effect of osmotic pressure can be ruled out. Root elongation was more affected than coleoptile growth. Less inhibition was observed with root exudates collected between the first and second and between the third and fourth leaf stages (Fig. 3), as compared with the effect of the same volume of root exudate (100 μl) collected between the emergence and the first leaf stage. The inhibition observed by the root exudate collected between the emergence and the fourth leaf stage could be accounted for by the sum of inhibitory effects of partial collections, indicating that as the collection time increases, the toxicity of the exudate also increases, due to an accumulation of allelochemicals in the collected exudates (Table 1).

Separation and identification of allelochemicals from wild oats root exudates

The elution pattern at 254 nm of a sample of wild oat root exudate continuously collected between the emergence and the first leaf stage is compared with its corresponding control (see Experimental) in Fig. 4a. Seven peaks were detected at this wavelength. Retention times of these peaks were compared with those of authentic phenolic acid and coumarin standards (Table 2). Scopoletin (20.9 μg), coumarin, *p*-hydroxybenzoic acid (5.2 μg) and vanillic acid were tentatively identified by this comparison. As the UV spectrum of scopoletin has a maximum at 340 nm, the same sample was run with the detector fixed at this wavelength and the chromatogram obtained was compared with that of the respective control (Fig. 4b). The elution pattern showed that the peak with the retention time of scopoletin had increased considerably in area. This wavelength was used to quantify scopoletin in root exudates of wild oats at different growth stages (Table 1). The results show that the amount of scopoletin trapped is higher in root exudates collected between the emergence and the first leaf stage than in root exudates collected between the first and second and between the third and fourth leaf stage. The results also showed that the amount of scopoletin trapped increased with the time of collection of root exudates. Other allelochemicals founded in the root exudates were not quantified as they were present in concentrations close to their limit of detection by HPLC.

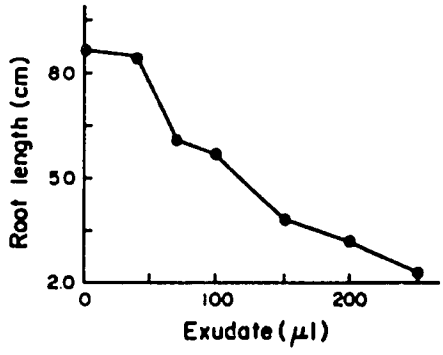

Fig. 1. Inhibition of root growth of spring wheat by root exudates of wild oat collected between emergence and first leaf stage, after 72 hr incubation in the dark at 21 ± 3°. LSD relative to control (5–11%).

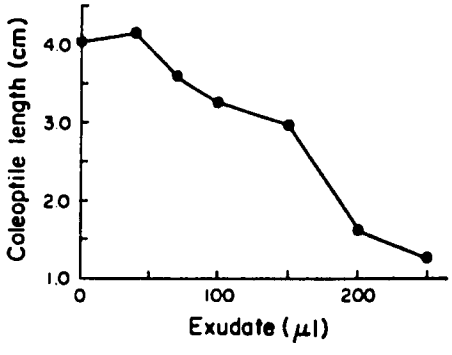

Fig. 2. Inhibition of coleoptile growth of spring wheat by root exudates of wild oat collected between emergence and first leaf stage, after 72 hr of incubation in the dark at 21 ± 3°. LSD relative to control (5–16%).

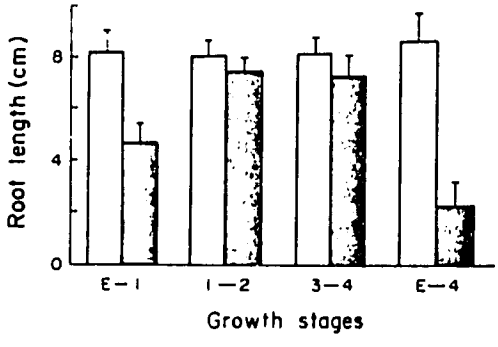

Fig. 3. Inhibition of root growth of spring wheat by 100 μl of root exudate of wild oat collected between different developmental stages of the plant (E = emergence, 1, 2, 3, 4 = first, second, third, fourth leaf stages) after 72 hr incubation in the dark at 21 ± 3°. Dark bars: root exudates; light bars: control collected without plants in the pot.

DISCUSSION

Collection of root exudates

Several methods have been employed to collect allelochemicals for bioassays and chemical identification from the undisturbed root system of plants. These methods include solvent extraction of hydroponic cultures [10, 11]

Table 1. Quantitation of scopoletin trapped from root exudates at different growth stages of wild oats

Growth stages	μg of scopoletin trapped*
E-1	20.9
1-2	6.4
3-4	7.3
E-4	34.8

*Scopoletin trapped from root exudates in pots containing 30 plants HPLC analysis was performed with the detector fixed at 340 nm.

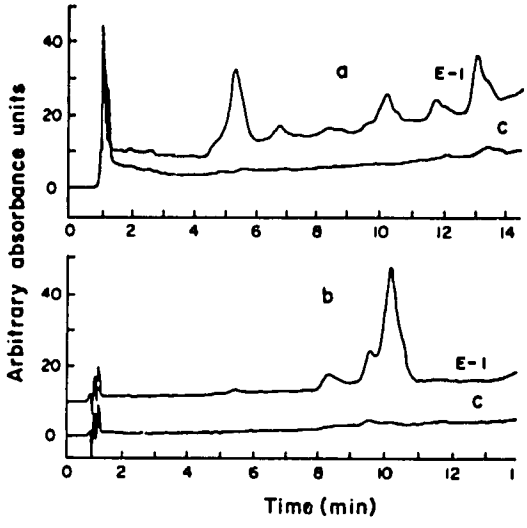

Fig. 4. HPLC chromatograms of root exudates of wild oat collected between emergence and first leaf stage (E-1) and of control (C) pots. Wavelength of the detector was fixed at 254 nm (a) and 340 nm (b).

Table 2. HPLC R_ts of standards and root exudates of wild oats

Standards	R_t* (min)	Peaks of root exudate†	R_t* (min)
Protocatechuic acid	3:19		
p-Hydroxybenzoic acid	5:15	1	5:18
Vanillic acid	6:40	2	6:44
Caffeic acid	7:08		
Syringic acid	7:33	3‡	8:16
Umbelliferone	9:13	4§	9:33
p-Coumaric acid	10:01		
Scopoletin	10:11	5	10:08
Salicylic acid	10:51		
Ferulic acid	11:07		
Coumarin	12:01	6	11:54
o-Coumaric acid	12:43	7‡	13:02
Cinnamic acid	16:36		

*See Experimental for HPLC conditions.
†See Fig. 4.
‡Unidentified peaks.
§Contamination peaks.

49

and washing of rooting medium such as sand [12]. Both methods are tedious and their success in the isolation of allelochemicals is limited [13]. The continuous root exudate trapping system developed by Tang and Young [9] overcame these difficulties using an Amberlite XAD-4 resin which retained hydrophobic compounds which were easily eluted with organic solvents. Control pots were submitted to the same conditions as planted pots. Bioassays using samples collected from control pots during one week period showed no growth inhibition of spring wheat seedlings, and HPLC analysis of these samples indicated the absence of significant contaminants. The use of vermiculite as the only inert medium in the continuous trapping system was the major difference with respect to the original method.

Identification of allelochemicals in root exudates of wild oat

Seven peaks were detected at 254 nm by HPLC when an RP C-18 column and a linear gradient of acidic water and methanol were used. When the UV detector was fixed at 340 nm, only three peaks were detected whose R_ts coincided with peaks 3–5 detected at 254 nm but with their respective areas increased indicating that at 340 nm the extinction coefficient was greater. Comparison of the R_ts of unknown peaks with phenolic and coumarin standards allowed the identification of *p*-hydroxybenzoic acid, vanillic acid, scopoletin and coumarin. Peaks 3 and 7 could not be identified. Peak 4 also appeared in the control experiment when the UV detector was fixed at 340 nm and was attributed to contamination. Scopoletin has been found in several cultivated oat accessions [13]. Recently, scopoletin and vanillic acid were tentatively identified in root exudates of wild oat collected by solvent extraction of the medium of hydroponic cultures [7].

Toxicity of wild oat root exudates

Root exudates from *A. fatua* collected between emergence and first leaf stage inhibited root and coleoptile growth of spring wheat seedlings. It has been reported that scopoletin inhibits seedling growth of tobacco (*Nicotiana tabacum*), sunflower (*Helianthus annuus*) and pigweed (*Chenopodium album*) at concentrations of 0.1 mM [14]. It has also been reported that tobacco and sunflower roots removed scopoletin from a nutrient medium with substantial quantities being translocated to the leaves [15]. The concentration of scopoletin present in our bioassays carried out with root exudates of *A. fatua* was *ca* 10 μM. Although the sensitivity towards scopoletin of spring wheat may differ from that of tobacco, sunflower and pigweed, it is likely that the concentration of scopoletin found is too low to produce the inhibitions observed. However, the presence of other allelochemicals in the root exudate could synergize the toxic effect of scopoletin as has been reported for other allelochemicals [16, 17].

The results presented suggest that allelopathy may be involved in the reduction of crop yields by infestation by wild oats, as exudates collected from the roots of wild oats are toxic to spring wheat seedlings. On the other hand, toxicity and the amount of scopoletin trapped in root exudates increased with the time of collection, suggesting that allelopathy could be an important factor in the interference of wild oats with wheat when the density of the weeds increased.

EXPERIMENTAL

Compounds. Standards listed in Table 2 were purchased from Sigma.

Seeds. Seeds of wild oats were obtained from the Quilamapu Agricultural Experimental Station, (Chillán, Chile). They were collected in 1986 and stored under dry conditions for two years. These seeds were non-dormant and had a germination rate higher than 95%. Seeds of spring wheat (cv Millaleu) were obtained from La Platina Agricultural Experimental Station, (Santiago, Chile).

Collection of root exudates. Seeds of wild oat were germinated in Petri dishes containing disks of Whatman N°1 filter paper. After radicle emergence, seeds were transfered to a continuous root exudate trapping system similar to that described in ref. [9]. The trapping system consisted of an inverted brown 5 l glass solvent bottle with the bottom removed. This pot was filled with a 6 cm layer of vermiculite. At the lower end of the bottle a column packed with Amberlite XAD-4 resin was attached, the output of which dripped into the top of the bottle. Circulation of H_2O and nutrient soln was assured by an air pump. The trapping action was based on the passage of cycling aqueous medium through the column and the elution of adsorbed chemical compounds from the resin by organic solvents. The system was sterilized before each use and covered with aluminum paper. Thirty seeds of wild oat were sown in the vermiculite. The system was kept in a chamber at 20 ± 5^c with a L12:D12 photoregime. The pots were irrigated with cycling distilled H_2O at a rate of 15 ml min^{-1} until the seedlings reached the first leaf stage. Then the column was detached and replaced by a new one and the plants were irrigated with Hoagland solution diluted 1:10, with H_2O [18]. This process was repeated until the fourth leaf stage of the plant was reached. In other experiments, the same column was maintained between the emergence and the fourth leaf stage of the plants. After collection, the Amberlite column was washed with 10 ml dist. H_2O and eluted with 100 ml MeOH. The MeOH soln was evapd to dryness under red. pres. and the residue redissolved in 1 ml MeOH and employed for bioassays and HPLC analysis. For each experiment the control consisted of a pot without seedlings and submitted to the same process as the sown pots.

Bioassays. Bioassays were carried out in Petri dishes containing one disk of Whatman N° 1 filter paper. Different volumes of trapped root exudates dissolved in MeOH were added to the filter paper. After solvent evapn, 3 ml dist. H_2O were added, and 10 seeds of spring wheat were placed on each disk. Root and coleoptile length were determined after 72 hr of incubation in the dark at 21 ± 3^c. Each bioassay was carried out in triplicates and the *t*-test was applied to determine significant differences.

HPLC analysis. Analysis was performed in a Knauer HPLC system equipped with two pumps type 364, mixing chamber, variable UV-Vis detector and HPLC-software package interfaced to a personal computer. Separation was carried out by using a reversed-phase Nucleosil 100 C-18 (4 × 120 mm) column with 5 μm particle size. Mobile phase was a linear gradient of H_2O (pH 3.0)-MeOH, 4:1 to 1:1, for 15 min and then H_2O-MeOH (1:1) for 5 min more. The flow was 1.2 ml min^{-1} and the eluate was analysed either at 254 or 340 nm. Samples were filtered through a 0.45 μm filter prior to injection. The vol. injected was 20 μl. Identification of compounds from the root exudates of wild oat was carried out by comparison of R_ts of unknown peaks with R_ts of authentic standards.

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51

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Hydroxamic acids affecting barley yellow dwarf virus transmission by the aphid *Rhopalosiphum padi*

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Key words: Wheat, *Triticum aestivum*, cereals, *Rhopalosiphum padi*, Aphididae, barley yellow dwarf virus, hydroxamic acids, DIMBOA

Abstract

2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), a hydroxamic acid (Hx) occurring in wheat, was shown to deter feeding by the aphid *Rhopalosiphum padi* (L.), and to reduce BYDV transmission to the plant. Dual choice tests with wheat leaves showed the preferential settlement of aphids on leaves with lower levels of DIMBOA. Electric monitoring of aphid feeding behaviour showed that in seedlings with higher DIMBOA levels fewer aphids reached the phloem and they needed longer times to contact a phloem vessel than in those with lower levels. When aphids carrying BYDV were allowed to feed on wheat cultivars with different DIMBOA levels, fewer plants were infected with BYDV in the higher DIMBOA cultivars than in the lower ones. Preliminary field experiments showed a tendency for wheat cultivars with higher Hx levels to be more tolerant to infection by BYDV than lower Hx level ones.

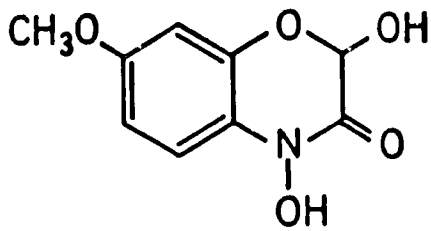
Introduction

The barley yellow dwarf viruses (BYDV) are members of the luteoviruses group affecting cereals (Oswald & Houston, 1953). As with other luteoviruses, they can only be transmitted by aphid vectors. These viruses are circulative within the vector, and are restricted to the phloem of the plant (Rochow & Duffus, 1981). Studies on BYDV transmission by *Sitobion avenae* (F.) to oat (*Avena Sativa* L.) showed that the vector needed plant access periods longer than a certain threshold in order to transmit the virus (Scheller & Shukle, 1986). The direct feeding damage by aphids has been diminished in Chile mainly via biological control (Zuñiga, 1985), but BYDV still remains a problem since virus transmission does not require a high population of the vector (Herrera & Quiroz, 1988). The principal BYDV strain present in Chilean crops is PAV (Herrera, 1984), which is transmitted by the aphids *S. avenae*, *Rhopalosiphum padi* (L.) and *Metopolophium dirhodum* (Wlk.) (Plumb, 1983).

The presence in the plant of resistance factors to the vectors might diminish the probability of virus transmission (Gibson & Plumb, 1977), in particular if such factors shorten the period during which virus may be inoculated. Hydroxamic acids (Hx) in cereals, and in particular 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) (Fig. 1) have been shown to play an important role in the resistance of the plant to pests and diseases (Niemeyer, 1988). The following results suggest that Hx may also be important in the prevention of virus transmission.

i) aphid survival on diets decreased as

57



DIMBOA

Fig. 1. Structure of DIMBOA.

DIMBOA concentration increased up to 4 mM; at higher concentrations survival increased eventually reaching the levels obtained with starved aphids. The interpretation of this phenomenon may be that at low DIMBOA concentrations aphids fed normally and ingested the compound, which produced its toxic effects decreasing aphid survival. As the DIMBOA concentration further increased, aphids were repelled by the compound, feeding was arrested and toxic effects were absent (Argandoña *et al.*, 1983).

ii) when aphids fed on diets with DIMBOA concentrations higher than 1 mM, the levels of DIMBOA in the aphids correlated negatively with the levels of the compound in the diets. A similar observation was made in aphids feeding on plants containing different DIMBOA levels (Niemeyer *et al.*, 1989b).

iii) studies on feeding behaviour of *Schizaphis graminum* (Rond.) showed that the average ingestion period for aphids feeding on diets with different DIMBOA concentrations, was inversely correlated with the levels of this compound (Argandoña *et al.*, 1983).

The purpose of this work is to examine the relationship between feeding deterrence by DIMBOA and BYDV transmission by aphids to wheat.

Materials and methods

Plant material. The cultivars employed were kindly supplied by Dr. Ignacio Ramírez and Ms. Mireya Zerené of the Instituto de Investigaciones Agropecuarias (INIA). Lines were classified by them as tolerant or susceptible to BYDV, as described (Herrera & Quiroz, 1988). In all the experiments carried out with the six wheat lines chosen (Platifén, Millaleu, Mexifén, Nobo, Anza and Maitén), plants were in the 1-leaf stage (G.S. 10, Zadoks *et al.*, 1974; Tottman & Makepeace, 1979). Seedlings show the highest levels of DIMBOA at this stage (Argandoña *et al.*, 1981).

Aphids. *R. padi* was collected in wheat fields near Santiago and kept in a rearing chamber at 22 ± 3 °C and a L16 : D8 photoregime. Aphids used in the experiments were apterous and differed in age by at most 24 h.

Choice experiments. Leaves of two cultivars grown on different pots were placed in a clip cage and 4 apterous aphid adults were introduced into the cage. 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.

Electrical penetration graphs (EPG). The system used has been described in detail (Tjallingii, 1988). The EPG technique consists of forming a closed circuit between plant and aphid by gluing one electrode to the aphid and placing another in the soil where the plant is growing. The circuit is closed when the aphid inserts its stylets into the plant. The signals produced are amplified and registered. For each cultivar studied, 20 apterous aphid adults were wired and their feeding behaviour recorded for 6 h. The mean time to reach the phloem and mean time ingesting from the phloem or from the xylem were determined. For 6 aphid adults on each line the recording was prolonged to 12 h and the mean duration of phloem sap ingestion was determined. Phloem sap ingestion was considered to occur when the characteristic EPG pattern developed for more than 8 min (Kimmins & Tjallingii, 1985).

Infection experiments. An aphid was allowed to feed for 6 h on wheat plants in the 1-leaf stage.

53

After that, the infectivity of each individual aphid was tested by transferring it to a single oat plant and killing it by spraying with insecticide 24 h later. Virus were allowed to develop for 1 month both in wheat and in oat. This experiment was carried out with twenty aphids for each of the six wheat accessions chosen. The presence of BYDV in wheat and oat plants was detected by means of an ELISA test (Lister & Rochow, 1979). Each set of experiments was repeated twice for each cultivar.

Controls. Oat was used as the control without hydroxamic acids in all the experiments described above. Results did not show significant differences with those obtained with wheat cultivar Platifén. Hence, they are not commented further.

Hx analysis. They were performed on wheat and oat plants at the 1-leaf stage, by high performance liquid chromatography (Niemeyer *et al.*, 1989a).

Results

DIMBOA levels in INIA lines. The accessions studied were classified by INIA as tolerant or susceptible to BYDV mainly on the basis of differences in yield of artificially infested vs. uninfested plants (Herrera & Quiroz, 1988). Figure 2 shows that most accessions classified as tolerant contained higher DIMBOA concentrations and most susceptible ones contained lower DIMBOA concentrations. Two exceptions occurred (lines 4 and 7), which probably present direct intrinsic tolerance towards the virus.

Choice tests. Choice tests with plants containing the same or different levels of DIMBOA showed that aphid distribution among high and medium or low Hx containing plants differed significantly, i.e. given the choice, aphids preferred to feed on plants with lower levels of DIMBOA (Table 1).

EPG's. Different signals in EPG's provided a means of recording the path of the stylet inside the plant leaf. Thus, it was possible to distinguish, among other variables, whether an aphid is probing or feeding in the phloem or in the xylem. The feeding time periods obtained in this way are shown in Table 2.

EPG's showed that i) aphids took longer to reach the phloem when the DIMBOA level in the plant was higher than when it was lower; ii) the higher the DIMBOA concentrations the lesser the number of aphids reaching the phloem; iii) once an aphid reached the phloem, its ingestion period did not vary significantly with Hx content.

Table 1. Effect of DIMBOA levels on aphids in feeding choice tests*

	Low Hx		Medium Hx		High Hx	
	(0.9)# Platifen	(1.0) Millaleu	(1.3) Mexifen	(1.4) Nobo	(2.0) Anza	(2.7) Maiten
Platifen	39 : *38*	34 : *28*	43 : *36*	33 : *28*	53 : *27**	42 : *21**
Millaleu		35 : *40*	37 : *31*	38 : *32*	56 : *24**	42 : *21**
Mexifen			36 : *36*	38 : *38*	39 : *28*	57 : *18**
Nobo				36 : *31*	44 : *28**	44 : *31**
Anza					26 : *26*	37 : *40*
Maiten						21 : *23*

* Paired values corresponds to cv at left: cv above.
\# mmol DIMBOA/Kg fr. wt.
* Differences in distribution are significant ($p(X^2) < 0.1$).

54

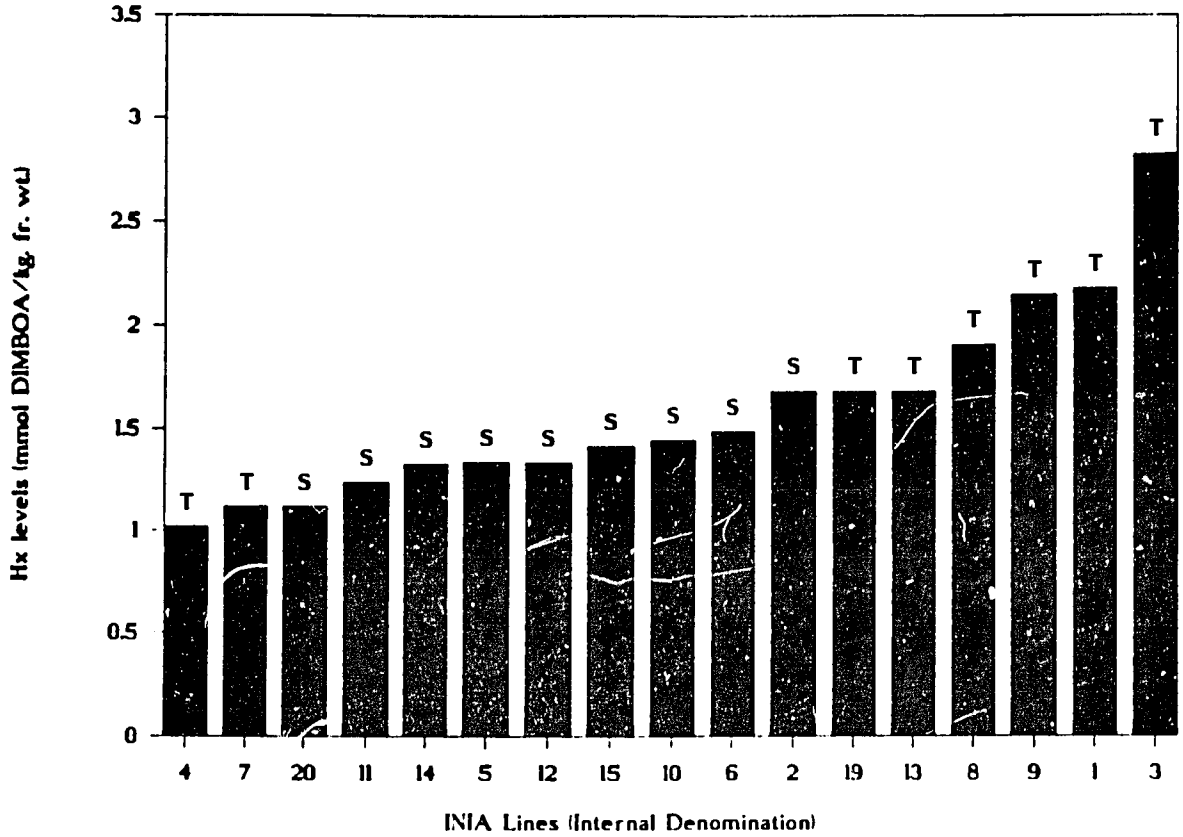


Fig. 2. Reaction to BYDV transmission on different wheat lines, and their hydroxamic acids levels (T = tolerant, S = susceptible).

Table 2. Effect of DIMBOA in wheat on aphid feeding

	Platifen	Millaleu	Mexifen	Nobo	Anza	Maiten
DIMBOA (mmol.kg fr. wt.)	0.9	1.0	1.3	1.4	2.0	2.7
% aphids arriving to phloem in 6 h	95	85	50	50	30	50
Time to phloem ingestion (min)	55a*	55a	67b	71b	95c	153d
Time in phloem (min)	353a	345a	342a	355a	343a	354a
Time in xylem ingestion (min)	34a	35a	50b	51b	77c	83c

* Values in the same row followed by the same letter are not significantly different at $p < 0.05$ (ANOVA/Duncan's test).

55

Table 3. Effect of DIMBOA on BYDV transmission by *R. padi*

	Platifen	Millaleu	Mexifen	Nobo	Anza	Maiten
DIMBOA (mmol/kg fr. wt.)	0.9	1.0	1.3	1.4	2.0	2.7
Nr of infected wheat plants	16	20	13	13	11	5
Nr of infected oat plants	18	20	20	18	20	16

Infection test. The infection results of Table 3 show a negative correlation between Hx content and BYDV transmission. Virus titres showed no significant differences among infected plants of the cultivars studied.

Discussion

The results obtained in choice tests (Table 1) supported the idea that aphids prefer those plants that contain lower levels of DIMBOA, suggesting that DIMBOA acts as a feeding deterrent in the plant. EPG's (Table 2) showed that this effect is perceived by the aphid during the period of stylet penetration, before reaching the phloem tissue.

On the other hand the mean time of phloem sap ingestion did not differ among the cultivars studied, suggesting that Hx are not present in the phloem sap or that they occur at concentrations below the feeding deterrent level (Niemeyer *et al.*, 1989b). These facts would imply that the threshold period of plant access needed for BYDV transmission, as postulated by Scheller and Shukle (1986), might be related to Hx levels, in the sense that these compounds would be a barrier for aphids before reaching a phloem vessel. Phloem sap analysis, for instance by means of a stylet excision technique (Weibull *et al.*, 1986), would give quantitative evidence of Hx presence or absence.

The results in Table 3 suggest that higher DIMBOA levels in wheat would interfere with BYDV transmission. Hence, Hx could be considered a resistance factor in wheat plants increasing the proportion of aphids searching for suitable feeding tissue by probing but not feeding, and dying or leaving the plant without transmitting virus, as described in general by Gibson and Plumb (1977).

It is known that alate aphids are important in the fitness of an aphid population due to their capability to migrate long distances and to decide whether a plant is or is not a suitable host for further colonization (Robert, 1987). Young apterous adults such as those used in our tests are less able to migrate long distances, but they also tend to move from plant o plant when the host suitability decreases (Klingauf, 1987). Although our choice tests (Table 1) would corroborate that apterous aphids are able to detect plant unsuitability, it seems desirable to develop similar experiments with alate *R. padi* in order to compare the behaviour of these two morphs. Since *Prunus padus* L., does not exist in Chile, this aphid could use wild Gramineae as host to overwinter (Carter *et al.*, 1980). Apterous morphs coming from an overwintering host and those coming from wheat crops may show differences in their behaviour. It would prove interesting to carry out comparative studies to test this idea.

Our results have been obtained with only one cereal aphid species. It would be desirable to carry out similar studies on other species such as *M. dirhodum* and *S. avenae* because they are able to transmit BYDV and show a differential sensitivity towards Hx (Corcuera *et al.*, 1982; Niemeyer *et al.*, 1989b).

56

These results were obtained with plants in the 1-leaf stage. As the plant ages, Hx content decreases (Argandoña *et al.*, 1981; Thackray *et al.*, 1990). However, they remain concentrated in the flag leaf (Thackray *et al.*, 1990; Leszczynski *et al.*, 1989). On the other hand, in Chilean crops it has been observed that the most important peak in aphid population occurs when the second node in wheat is noticeable (Herrera & Quiroz, 1988). To project our results to field conditions it would be desirable to develop cultivars possessing high Hx levels throughout the life of the plant or at least at growth stages when aphid populations are high. A possible role of Hx preventing BYDV transmission by aphids in older plants, at the time when natural populations are high under field conditions can be deduced from the results reported in this work (Fig. 2). Thus, with two exceptions, plants classified as tolerant to BYDV showed higher levels of Hx than those which were classified as susceptible to the virus.

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-59

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58

MECHANISMS OF RESISTANCE IN CEREALS TO PESTS

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ABSTRACT

A research programme to detect resistance to pests in a crop should ideally address at the outset the following questions: How wide a genetic range of plant material should be screened? Should the mechanisms of resistance be investigated? Should the biological components (antibiosis, antixenosis, tolerance) be studied? At what point should plant breeders be involved? An alternative to the above approach is the crude but rapid screening of large numbers of plants by releasing pests and assessing plant survival after an interval. Examples of both approaches are given, concentrating on cereals, with a discussion of the advantages and limitations of each strategy.

INTRODUCTION

Host plant resistance to insects has the potential, acting alone, or with insecticides and/or with natural enemies, to keep pest numbers below economic thresholds and thereby reduce pesticide inputs. Pesticides are not always used rationally on U.K. cereals, for instance; economic losses (Wratten & Mann, 1988; Wratten et al., 1990) and undesirable side-effects can occur even when products are applied in response to spray thresholds (Vickerman & Sunderland 1987; Vickerman et al., 1987; Sotherton et al., 1987; Wratten et al., 1988). In addition to the above economic and environmental advantages, simple cost-benefit analysis relating research and development costs for a new resistant cultivar to its economic value following release can be 1:300 (Luginbill, 1969). The development of a typical new pesticide, however, may incur an expenditure of $50m, with little prospect of recouping this outlay for many products before patent expiry (Finney, 1988). Because of these high costs, return on investment has been delayed, raising doubts in some companies about long-term investment in agrochemical R & D (Finney, 1988). Against this must be weighed the limitations of host-plant resistance as a major pest-control method. These limitations will be discussed in this paper, which will use case studies of cereal pests to illustrate prospects for the use of host plant resistance.

APPROACHES TO THE INVESTIGATION OF RESISTANCE

Screening via crude bioassays

The simplest way of searching for useful levels of resistance is to inundate a wide range of replicated plant material in the laboratory, glasshouse or field, with large numbers of the pest and leave the system for

54

a few days or weeks. All that need be assessed after the chosen interval has elapsed is the condition and number of surviving plants. In theory, this method can identify highly resistant lines which survive the high pest pressure and has been used for aphids by Reinink & Dieleman (1989) in a glasshouse screen of lettuce cvs., by Birch (1989) for turnip root fly (Delia floralis) in brassicas, and by Dunbier, Kain & McSweeney (1977) in New Zealand; the latter led to the release of a new, aphid-resistant lucerne cultivar within three years of initial screening. Although this method is intuitively attractive, it does have potential drawbacks, especially if the initial screening is not conducted under realistic conditions. The main limitation is that no information on the classical components of resistance (antibiosis, non-preference, tolerance; Painter, 1951) is obtained, or on resistance mechanisms. This also means that screening has to be based on bioassays using the pest, rather than via a short-cut involving screening for cuticular hair density, levels of secondary plant compounds, etc. Also, should the resistance break down in the field, the lack of a detailed understanding of its nature means that an explanation for the breakdown is unlikely to be found. Because of this method's broad approach, however, it has the advantage that it is not biased in favour of only one component; some laboratory screens concentrate on antibiosis only, for instance the work by Bohidar et. al., (1986) and Spiller & Llewellyn (1987) on seedling resistance in wheat to aphids.

Evaluation of the biological components and mechanisms of resistance

The advantage of investigating all potential resistance components is that there is less likelihood that a key insect-plant interaction will be overlooked. If, in addition, a major mechanism of resistance is identified, the possibility exists for a rapid biochemical or physical (e.g. leaf hairs) screen of a very large range of plant material without the time-consuming elements of insect culturing and bioassays. Of the three components proposed by Painter (1951), antibiosis (defined in this case as an effect on the performance of the insect, via fecundity, mortality, development rate, etc.) is the commonest in the literature, especially among laboratory studies. It often involves the caging of single insects on plants, and in the case of aphids on cereals, of recording such parameters as intrinsic rate of increase (r_m; Birch, 1948), relative growth rate (van Emden, 1969), rate of honeydew production (Spiller & Llewellyn, 1987), number of embryos in the adult parthenogenetic female (Bintcliffe & Wratten, 1982), adult weight (Lee, 1983) etc. Host non-preference (= antixenosis) is a less relevant measurement for laboratory screening since it is likely to be greatly influenced by environmental conditions in the field, including the crop's agronomy and the areas of monoculture over which it is grown. The relevance of simple laboratory choice experiments involving cereal aphids (e.g. Leather & Dixon, 1982; Dent, 1986; Dent & Wratten, 1986) to single-cultivar monocultures is questionable. Tolerance, whereby certain cvs. yield better than do others per unit pest number, is a realistic measurement only under field conditions. Even then, unless it operates below or near the pest's economic threshold, it would be unlikely to replace or even supplement pesticide use. Although tolerance is unlikely to promote the development of resistance - breaking pest biotypes, if employed as the major component it could lead to high pest levels on a regional basis, leading to future problems.

Screening of a wide genetic range of plant material

Concentration on commercial cvs. has the potential disadvantage that they may have low levels of resistance compared with their ancestors and wild relatives. This decline in resistance with cultivation history is well

60

illustrated by the black bean aphid, Aphis fabae on Vicia (Fig. 1). The disadvantage of screening plant material which is genetically remote from commercial cultivars is that crosses may be difficult to make, due to 'premating' and/or 'postmating' problems (Stalker, 1980).

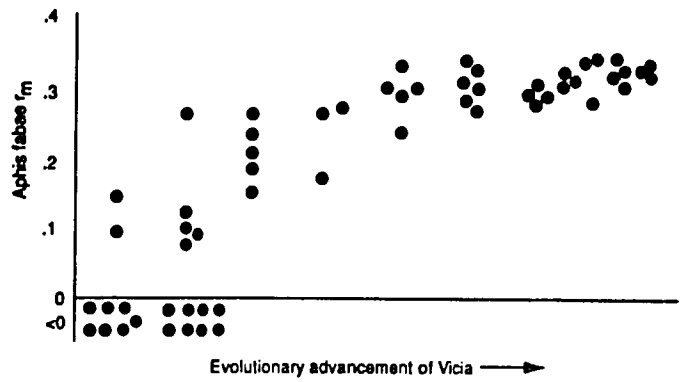

FIGURE 1. Performance of Aphis fabae in relation to evolutionary advancement of Vicia hosts (from Holt & Birch, 1984)

CASE STUDIES OF RESISTANCE IN CEREALS

The role of hydroxamic acids in conferring resistance to Lepidoptera and aphids

The structure of hydroxamic acids (Hx) is similar to that of amides with a hydroxyl group rather than a hydrogen atom attached to the nitrogen atom. Hx from cereals are benzoxazinone type cyclic Hx, i.e. benzene ring is fused to a 6-membered ring containing an oxygen atom besides the hydroxamic acid function. They share much of their biosynthetic pathway with tryptophan. DIMBOA (2, 4-dihydroxy-7-methoxy-1, 4-benzoxazin-3-one) is the most abundant compound among the Hx which have been isolated from maize, wheat and several related wild Gramineae. They are found in the plant as glucosides which are enzymically hydrolysed to the corresponding aglucones when the plant tissue is injured (see review by Niemeyer, 1988).

Early work on Hx concentrated mainly on resistance in maize. Klun, Tipton & Brindley (1967) reported significant correlations between resistance to the European corn borer (Ostrinia nubilalis) and levels of benzoxazolinones (hydroxamic acid breakdown products) in maize extracts and followed this with bioassays using artificial diets which showed hydroxamic acids to be the most active plant component. Robinson, Klun and Brindley (1978) demonstrated feeding deterrent effects both in field and laboratory tests. In the 1980's, recurrent selection based either on DIMBOA levels or on insect damage led to improved first-brood resistance.

Long, Dunn, Bowman & Routley (1977) reported a highly significant negative correlation (r = 0.72) between the level of infestation of the corn leaf aphid (Rhopalosiphum maidis) and the concentration of DIMBOA in maize tissue. Argandona, Luza, Niemeyer and Corcuera (1980) found similar

- 61

relationships for the rose-grain aphid Metopolophium dirhodum on wheat. I addition hydroxamic acids have been implicated as resistance factors i several wheat cultivars to the aphids Schizaphis graminum and R. maidi (Corcuera, Argandona & Niemeyer, 1982). S. avenae was investigated in thi context in preliminary work by Bohidar et. ai. (1986) who found that 96% o the variance in the resistance of six Chilean wheat cultivars was explaine by Hx levels. Although Hx levels decline as plants age (Thackray et. al 1990), the youngest leaves of seedlings had high Hx levels, while the level in young flag leaves were similar to those of some seedlings. The latte data are important as S. avenae causes yield loss when feeding on flag leave and ears of mature wheat plants (Wratten, 1978) and is not a seedling pest i the U.K. other than as a BYDV vector in N. England (McGrath & Bale, 1989) Leszczynski et. al. (1989) demonstrated a highly significant negativ correlation ($r = -0.905$) between S. avenae intrinsic rate of increase (r_m and Hx concentration in flag leaves of wheat. Screening mature plants for H or via an insect bioassay is time-consuming, however, as at least two month are needed to obtain wheat in ear under U.K. glasshouse conditions. If ther was a correlation between seedling Hx or aphid resistance and their values i mature plants, this would be of value in accelerating a screening programme In a series of papers by Lowe in the 1980s (e.g. Lowe, 1984 a,b,c) aphi bioassays were used to rank a large number of cvs. of winter wheat i relation to their resistance as semi-mature plants to the grain aphid. Whe these were evaluated as two-leaf seedlings for DIMBOA content, using hig performance liquid chromatography (Fig. 2 a,b; see Niemeyer et. al., 1989 fo methods), a wide range of DIMBOA content was obtained (Fig. 3). Resistanc to the grain aphid was measured in six of these cvs. which showed th greatest range in Hx concentration and the correlation was very high ($r = -0.94$; Fig.4). However, there was no significant correlation between th rankings of these cvs. as seedlings, based on Hx levels and aphid relativ growth rate, and the rankings of Lowe(loc. cit. There was also no significant correlation between the Hx ranking of all 36 cvs. as seedlings and their ranking in Lowe's mature plant bioassays. Hydroxamic acids can therefore explain a high proportion of the variation in resistance to S. avenae in wheat, but plants need to be screened for Hx levels and resistance at the same growth stage. The effect is mainly via antibiosis, although the fact that inverse relationships exist between DIMBOA levels in aphids feeding on wheat p lants and DIMBOA levels in the plants themselves suggest that feeding deterrency may be involved (Niemeyer et. al., 1989).

61

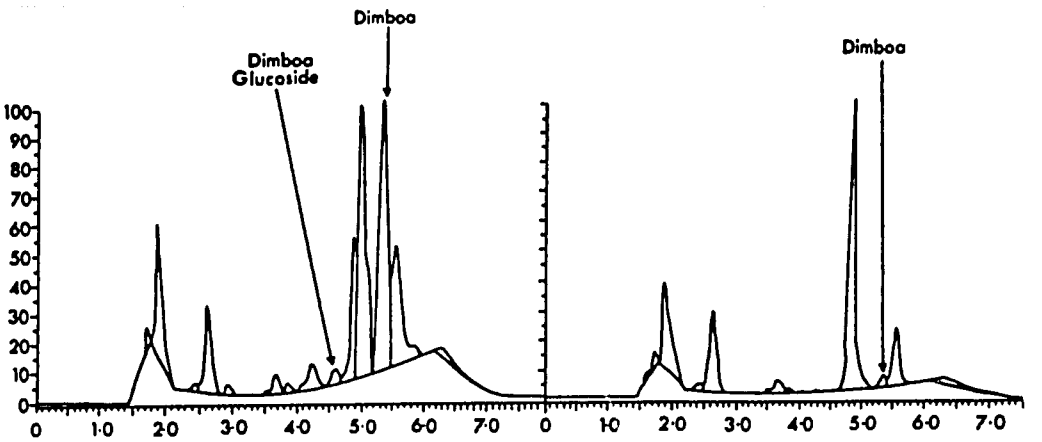

FIGURE 2. HPLC analyses of high and low DIMBOA wheat cultivars.

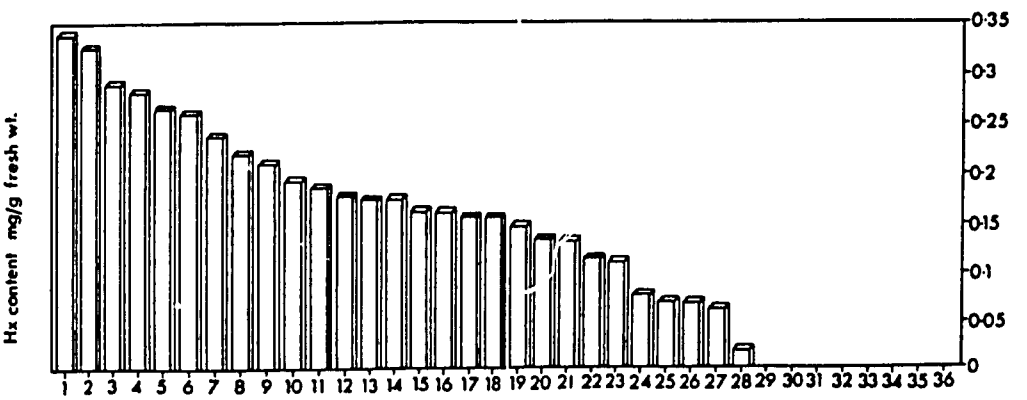

FIGURE 3. Range of DIMBOA concentrations in seedlings of cultivars screened by Lowe as mature plants via aphid bioassays.

The role of physical factors in aphid resistance in wheat

Lowe *et al.* (1985) demonstrated a role for antixenosis in wheat against *S. avenae*, with the possibility that non-glaucous cvs. may be avoided by the aphid. Acreman & Dixon (1986) examined six genotypes of awned spring wheat and showed that aphids were up to 22% less fecund, and were more likely to be dislodged than aphids feeding elsewhere on an ear. These two factors reduced aphid population growth to one third of that on awnless plants. Population modelling by Acreman (1984) revealed that with the regular reductions in aphid numbers of cereals by 85% which are feasible via host plant resistance, the three outbreaks recorded over a five-year study period would not have occurred.

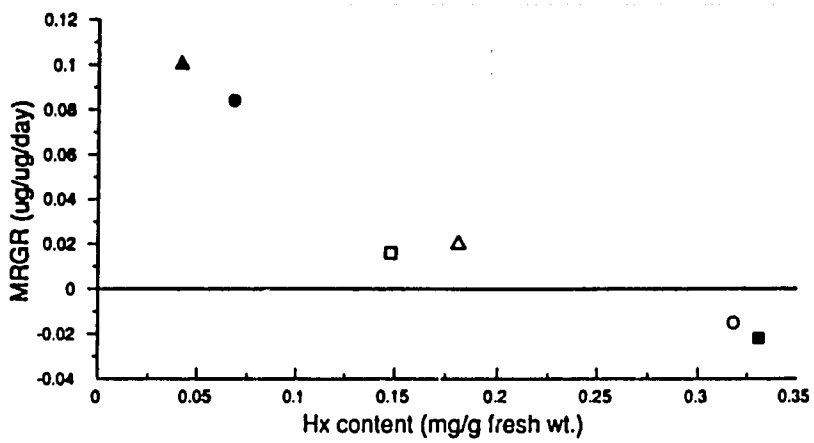

FIGURE 4. Relationship between mean relative growth rate of Sitobion avenae and seedlings selected from those in Fig. 3 representing a high range in hydroxamic acids.

Resistance to aphids as virus vectors

This is usually biologically less tractable than is the production of resistance against direct-damaging pests as resistance is usually only partial and thresholds for virus vector control are usually low. However, Givovich & Niemeyer (1991) showed that in wheat seedlings with high DIMBOA levels, fewer plants were infected with BYDV by R. padi than on low DIMBOA cvs. Fewer aphids reached the phloem on the high DIMBOA cvs. and those which did reach the phloem took longer on high DIMBOA cvs. In W. Europe, R. padi feeds low down on wheat and barley seedlings, often on the leaf sheaths at or below ground level. In view of the above results for S. avenae (Fig. 4; which showed the importance of evaluating the appropriate growth stage, it is relevant to examine leaf sheath Hx levels in seedling wheat. This was done for R. padi on a range of Triticum material, using relative growth rate and HPLC analysis of DIMBOA, as described above. A correlation coefficient of - 0.794 was obtained (Fig. 5). This evidence, and that of Givovich & Niemeyer (1991) suggests that Hx levels may be a good indicator of pest performance in plant breeder's screening, not only for direct-damaging pests such as S. avenae but for virus-vectors such as R. padi.

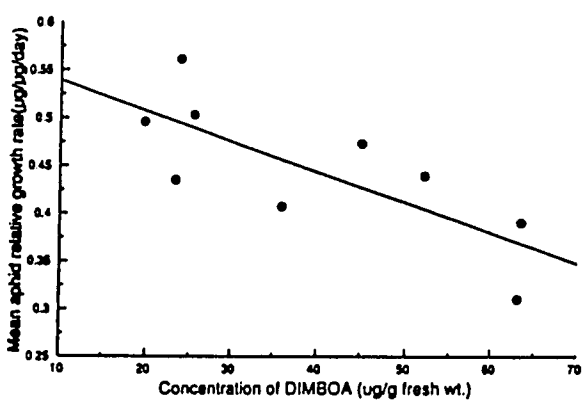

FIGURE 5. Relationship between mean relative growth rate of Rhopalosiphum padi and seedling sheath hydroxamic acid levels.

Resistance to other cereal pests

Silica has been implicated in the resistance in grass to frit fly (Oscinella frit) attack (Moore, 1984), and the distribution and quantity of silica within the plant may make a major contribution to control of this fly in grassland (Clements & Henderson, 1983). Silica was also implicated in resistance in oats and wheat to Hessian fly (Mayetiolia destructor; Miller et. al., 1960), and in rice resistance to mollusc grazing (Wadham & Wynn-Parry, 1981). The addition of sodium silicate to host plants reduced growth rates of populations of frit fly and S. avenae (Hanisch, 1981). The structure and persistence of the coleoptile (in oats) and colour (in ryegrass) determine levels of oviposition by the frit fly.

CONCLUSIONS AND PROSPECTS

Even a relatively small increase in resistance can reduce the probability of a pest outbreak, especially in combination with the action of natural enemies with or without insecticide use (see van Emden, 1987 and van Emden & Wratten, 1990 for reviews). However, Burn (1987) in a review of integrated pest management in cereals, concluded that for most insect pests in these crops the ' ... use of varietal resistance seems remote, and will remain so while so little attention is given to it in plant breeding programmes'. This is in contrast to resistance to fungal pathogens, where varieties exist which offer some resistance to eyespot, powdery mildew, Septoria and yellow and brown rust. It is interesting to speculate whether the absence of aphid-resistance breeding programmes leading to the growing of aphid-susceptible cvs. (such as Maris Huntsman in the 1970s) over large areas, may have influenced grain aphid abundance at field or regional 'meta-population' (Hanski, 1989) scales. The reason for this lack of progress in insect resistance is likely to be a combination of a) arable pests are easily controlled chemically, with no serious insecticide resistance in cereal pests b) there has been no suitable biochemical or physical factor in cereals which could be used as an indicator of pest performance, to speed up the screening process c) bioassays were often laboratory based and concentrated on modern cvs. which did not reveal large differences in resistance for use in breeding programmes d) environmental awareness of insecticide side-effects has been much higher recently than in the 1970s, leading to moves in some European countries to reduce these inputs substantially.
e) some plant breeding concerns have been taken over by agrochemical companies; this may make it less likely that pest-resistant cvs. will be produced from this source as insecticide sales may suffer as a consequence.

Despite the above five partly historical reasons, in the 1980s and 1990s a general desire to reduce pesticide inputs has arisen alongside the demonstration that some physical (e.g. awns) and biochemical (e.g. DIMBOA) factors can explain high proportions of the variation in aphid numbers on wheat. It seems that it is now timely to exploit this information in directed screening and plant breeding programmes, possibly via technological development agencies such as the British Technology Group in the U.K. or via agrochemical companies.

ACKNOWLEDGEMENTS

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DIFFERENCE IN HYDROXAMIC ACID CONTENT IN ROOTS AND ROOT EXUDATES OF WHEAT (*Triticum aestivum* L.) AND RYE (*Secale cereale* L.): POSSIBLE ROLE IN ALLELOPATHY

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Abstract—Hydroxamic acids (Hx) produced by some cereal crops have been associated with allelopathy. However, the release of Hx to the soil by the producing plant—an essential condition for a compound to be involved in allelopathy—has not been shown. GC and HPLC analysis of roots and root exudates of wheat (*Triticum aestivum* L.) and rye (*Secale cereale* L.) cultivars, with high Hx levels in their leaves, demonstrated the presence of these compounds in the roots of all cultivars analyzed and in root exudates of rye. Moreover, bioassays employing root exudates collected from wheat and rye seedlings demonstrated that only rye exudates inhibited root growth of wild oats, *Avena fatua* L., a weed whose root growth is inhibited by Hx. These results suggest that rye could potentially interfere with the growth of *Avena fatua* in nature and that this interference could be due to the release of Hx to the soil by way of roots.

Key Words—Hydroxamic acids, root exudates, allelopathy, *Triticum aestivum* L., *Secale cereale* L., wild oats, *Avena fatua*.

INTRODUCTION

Hydroxamic acids (Hx) isolated from wheat and other cereals, have been proposed to impart plant resistance to pests and diseases (Niemeyer, 1988). Hx

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169

isolated from rye, 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), and its decomposition product, benzoxazolin-2-one (BOA), have been implicated as potential allelopathic agents of residues of this crop (Barnes and Putnam, 1987; Barnes et al., 1987). Recently, it was reported that these compounds are decomposed by microorganisms in the soil to 2,2'-oxo-1,1'-azobenzene (AZOB) or its methoxy derivative (MAZOB) and that these transformed compounds have stronger herbicidal activity than the original molecules (Nair et al., 1990). Previous results obtained by us indicated that 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), the main Hx isolated from wheat, and its decomposition product, 6-methoxybenzoxazolin-2-one (MBOA), inhibit root growth of wild oats (Pérez, 1990), a weed that normally infests wheat fields (Bell and Nalewaja, 1968; Cudvey et al., 1989).

One of the essential features of allelopathic interactions between plants is that phytotoxins or substrate for phytotoxins must be released from a plant to the soil and absorbed by a second plant (Rice, 1984). In this paper we present data on Hx levels in roots and root exudates of three different wheat cultivars and two rye cultivars. The presence or absence of toxicity of root exudates collected from wheat and rye plants was assessed with wild oat bioassays.

METHODS AND MATERIALS

Reference Compounds. DIMBOA was isolated from ether extracts of *Zea mays* L. cv. T129 s, as described by Queirolo et al. (1983). DIBOA was synthesized as described by Jernow and Rosen (1975).

Seeds. Seeds of spring wheat cultivars Andifén, Alifén, and Likay-INIA and of wild oats (*A. fatua*) were obtained from the Agricultural Experimental Station, La Platina (INIA). Seeds of rye cultivar Forrajero Baer and Tetra Baer were obtained from E. von Baer Seeds Co., Temuco, Chile.

Collection of Root Exudates. Seeds of wheat and rye cultivars were germinated in Petri dishes containing Whatman No. 1 filter paper. After radicle emergence, seeds were transferred to a continuous root exudate trapping system using vermiculite as an inert medium (Tang and Young, 1982). The system was autoclaved for 40 min at 15 psi before use and covered with aluminum foil. Thirty seedlings of each cultivar were planted in vermiculite and grown in a light bank at 20 ± 5°C and a 12 light-12 dark photoregime. Each pot was irrigated continuously by recycling distilled H_2O at a rate of 15 ml/min until the seedlings reached the first leaf stage. At this point, the Amberlite column was removed from the system and washed with 10 ml of distilled H_2O. Adsorbed organic materials were eluted from the column with 100 ml MeOH. The MeOH solution was evaporated to dryness under reduced pressure at a temperature of 35°C, and the residue was redissolved in 1 ml MeOH. This latter solution was

-10

employed for the bioassays, HPLC, and GC analysis. Control solutions were obtained by the same procedures from systems without seedlings.

Root Extracts. Once the seedlings reached the first leaf stage, roots were removed from the vermiculite in the root exudate system and washed with distilled H_2O. Root tissue (100–150 mg) was macerated successively with 3 × 0.33 ml of distilled H_2O. The extract was adjusted to pH 3 with 0.1 N H_3PO_4 to avoid Hx decomposition and then centrifuged at 6000 *g*. The resulting supernatant was analyzed by HPLC (Niemeyer et al., 1989).

HPLC Analysis. Analysis was performed in a Knauer HPLC system equipped with two pumps (type 364), mixing chamber, variable UV-Vis detector and HPLC software package interfaced to a personal computer. Isolations of the compounds of interest were obtained by using a reverse-phase Zorbax ODS C-18 (4.6 × 25) column with a 5-μm particle size. A linear gradient of H_2O (pH 3)-MeOH, 80:20 to 50:50 for the first 10 min with an additional 20 min at 50:50 was used. The flow was 1.0 ml/min, and the eluate was analyzed at 254 nm. Samples were filtered through a 0.45-μm filter prior to injection. The volume injected was 20 μl. Concentrations of DIBOA and DIMBOA in the samples were obtained by extrapolation from the corresponding calibration curves obtained with pure compounds.

GC Analysis. Aqueous root extracts (1 ml) were evaporated to dryness and redissolved in 100 μl MeOH. Fifty microliters of this solution were derivatized with 50 μl of *N, O*-bis(trimethylsilyl) acetamide for 20 min at 50°C. Methanolic solutions of root exudates and standard samples were derivatized in the same way. GC analysis was performed in a Shimadzu GC-PA system equipped with a capillary column and a FID detector. The N_2 was used as carrier gas and the injector and detector temperatures were 250°C. Samples were run with a temperature gradient between 180 and 206°C at a rate of 8°C/min.

Bioassays. Bioassays were carried out in Petri dishes containing Whatman No. 1 filter paper. Different volumes of root exudates dissolved in MeOH ranging from 100 to 250 μl were added to the filter paper. After solvent evaporation, 3 ml of distilled H_2O were added and 10 seeds of wild oats were placed on each disk. Root lengths were measured after 72 hr of incubation in the dark at 20 ± 3°C.

RESULTS

HPLC and GC Analysis of Hx in Roots and Root Exudates of Wheat and Rye Plants. Figure 1 depicts typical HPLC chromatograms of root extracts and root exudates from wheat and rye plants. The peak at 8:06 ± 0.05 min was identified as DIBOA and the peak at 10:15 ± 0.15 min was identified as DIMBOA. Identification was based on retention times and UV spectra of authentic

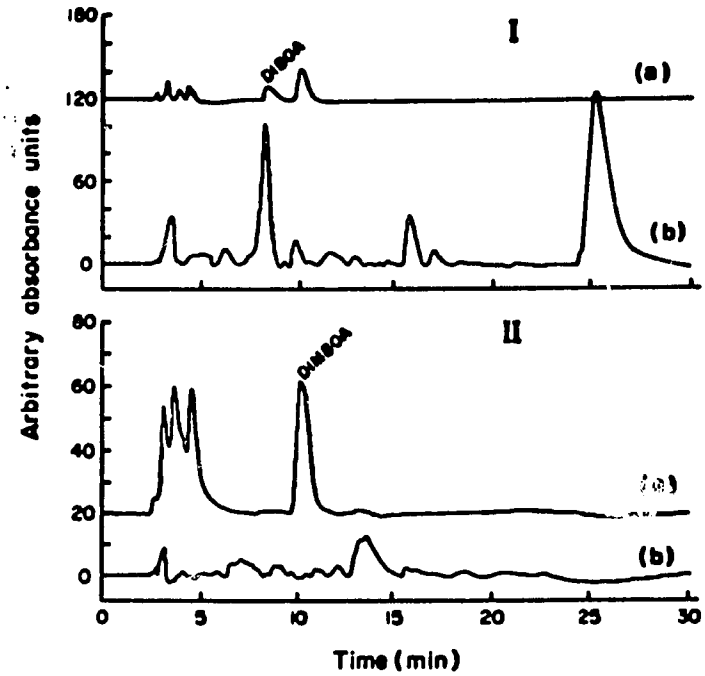

FIG. 1. HPLC chromatograms of root extracts (a) (100–150 mg tissue in 1 ml H_2O) and root exudates (b) (collected from 30 plants and dissolved in 1 ml MeOH), of rye cultivar Forrajero Baer (I) and wheat cultivar Andifén (II).

standards. The same samples were also analyzed by capillary GC and its retention times compared with those of DIBOA 3.1 ± 0.01 min and DIMBOA 5.0 ± 0.01 min. In the HPLC chromatogram of root extracts from rye, a peak appeared with a similar retention time as DIMBOA, but the UV spectrum of this peak did not correspond with the standard nor was DIMBOA observed in the GC chromatogram.

In root extracts, DIBOA was detected by HPLC and GC in wheat and rye cultivars, while DIMBOA was detected only in wheat cultivars. In root exudates, DIBOA was the only Hx detected, and it was found only in rye (Table 1).

Bioassays. Root exudates collected from the wheat cultivar Andifén and from the rye cultivar Forrajero Baer between emergence and the first leaf stage were tested for toxicity on seeds of *A. fatua*, a weed whose root growth is inhibited by Hx (Pérez, 1990). The results (Figure 2) demonstrate that only root exudates collected from rye inhibited root growth of wild oats and that the level of inhibition was determined by the volume of exudate tested.

-72-

TABLE 1. QUANTITATION OF HYDROXAMIC ACIDS IN ROOT EXTRACTS AND ROOT EXUDATES COLLECTED BETWEEN EMERGENCE AND FIRST LEAF STAGE FROM WHEAT AND RYE CULTIVARS

Cultivar	Root extracts (mmol/kg fr wt)[a]		Root exudates (μmol/kg fr wt)	
	DIBOA	DIMBOA	DIBOA	DIMBOA
Rye				
Tetra Baer	0.8	nd[b]	0.07	nd
Forrajero Baer	1.3	nd	25.0	nd
Wheat				
Alifén	0.9	2.3	nd	nd
Andifén	nd	0.8	nd	nd
Likay-INIA	0.01	1.6	nd	nd

[a]Root fresh weight of plants at the first leaf stage.
[b]nd = not detected.

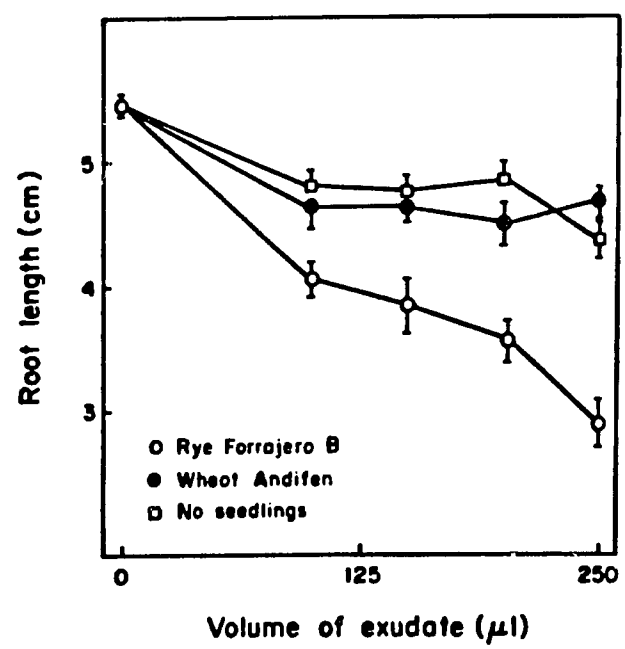

FIG. 2. Radicle lengths of wild oats (*A. fatua* L.), treated with different amounts of root exudates collected from wheat cultivar Andifén (●), rye cultivar Forrajero Baer (○) and without seedlings (□). Data are mean ± SE.

DISCUSSION

Root Exudates. It has been known for many years that organic compounds are exuded from healthy undamaged plant roots (Rovira, 1969). "Normal exudates" are thought to be composed of soluble, low-molecular-weight compounds that are mixed in the rhizosphere with root mucilage, root cap cells, dead root hairs, and epidermal cells (Nye and Tinker, 1977). Thus the distinction between "normal exudates" and other organic materials in the rhizosphere is unlikely even in hydroponic cultures (Barnes et al., 1986) and washings of rooting medium such as sand (Abdul-Wahab and Rice, 1967) include materials that were initially part of the plant structure. However, the results in Table 1 show that hydroxamic acids retained by the XAD-4 column are not a product of cell rupture; i.e., they are "normal exudates," since the levels found in the rhizosphere do not correlate with levels in the roots. Thus, it would seem plausible that exudation of hydroxamic acids is an active process.

Specifity of Root Exudates. DIBOA was the only Hx detected in root exudates and was found only in rye. The amount of DIBOA exuded by rye plants is not proportional to its content in the roots. Additionally, the wheat variety Alifén contained both Hxs in its roots and none of them was exuded. These results suggest that root exudation of Hx depends on a specific regulating factor that is not related with either concentration or type of Hx present in the root tissue.

These observations suggest that simply identifying roots with high contents of Hx is not adequate for the selection of varieties with allelopathic potential. Root exudate analysis will also be required. It has been reported that stress and other factors such as plant age, plant nutrition, light, and moisture can greatly increase root exudation (Nye and Tinker, 1977). The assessment of these factors, together with the search for rye germplasm with a high capacity to exude Hx by its roots, are of interest relative to the potential of Hxs as weed inhibitors through root exudation.

Toxicity of Root Exudates. In a previous paper we demonstrated that Hx can inhibit root growth of wild oats (Pérez, 1990), a weed normally associated with wheat. On the other hand, it has been suggested that rye interferes with the growth of wild oats among other plants (Barnes et al., 1986). The findings that Hx is not lost from roots and is not leached from leaves (Argandoña et al., 1987) of wheat suggest that Hx is not an important allelopathic agent associated with living wheat plants. The detection of DIBOA in root exudates of rye plants and that these root exudates inhibited the growth of wild oats suggest that Hx or its transformation products (Nair et al., 1990) may be important in inhibiting the growth of wild oats in nature.

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SECONDARY PLANT CHEMICALS IN APHID-HOST INTERACTIONS

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Introduction

Aphids damage plants by draining nutrients from them, by transmitting viral diseases, by inoculating toxins through their saliva, and by interfering with photosynthetic efficiency and providing a rich medium for fungal growth through their excretion products (Schepers 1989).

Control measures for aphids include, in the case of heteroecious species, measures at the primary host such as early defoliation, oil spray, pruning, and preservation of a habitat for predators. Migration to the secondary host may be prevented by the control of weeds that may act as a bridge, the application of behavior-modifying chemicals, and various ways of host plant masking. At the secondary host, control may be exerted by variations in the quality of the host, which may be achieved by manipulation of its nutrient value or its content in allelochemicals, commonly secondary plant metabolites (Harrewijn and Minks 1989), among other methods (van Emden and Wratten, this volume).

This paper deals with the role that secondary metabolites from plants play in aphid-plant relationships. Emphasis will be placed on the involvement of hydroxamic acids from cereals in aphid resistance.

Host Finding by Aphids: Sensing Plant Secondary Metabolites

The finding of the host by an aphid consists of a series of events, as summarized in Figure 1. Arrival at an area with potential host plants is a chance event depending to a large extent on aphids being carried by wind. Once in the neighbourhood of potential host plants, visual and olfactory

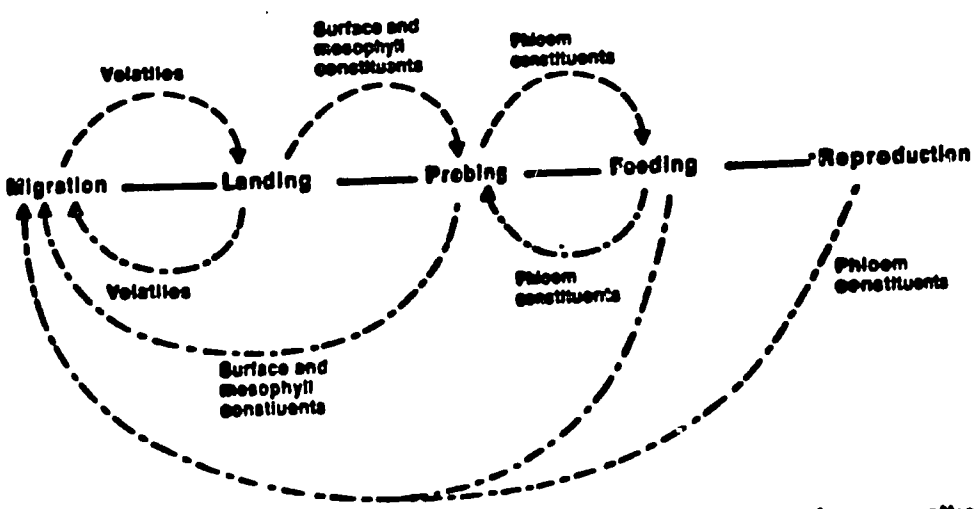


Fig. 1. Host finding by aphids: intervention of plant secondary compounds. - - - = attractive compounds or feeding stimulants; — · — = repellent, antifeedant or antibiotic compounds.

orientations play a more important role. Visual organs are present in all instars, and secondary compound eyes, which are more developed in adults, contain ommatidia which tend to be larger and more numerous in alatae, consistent with their migratory and host searching activities (Anderson and Bromley 1987). The number of ommatidia is limited and hence visual stimuli play a role only at close range (Harrewijn and Minks 1990). Olfactory organs are present on the antennae. Their number is also limited and thus olfaction also plays a role only at close range (Anderson and Bromley 1987).

After aphid alightment on a plant, surface chemicals become important in host acceptance. These may be components of the epicuticular lipid layer of the plant (Dillwith and Berberet 1990) or components in the trichomes of certain plant species (Gibson and Pickett 1983; Avé et al. 1987). The nature of these chemicals is perceived by chemoreceptors on the antennae (Bromley et al. 1979) and on the apex of the tibiae and the tarsi (Anderson and Bromley 1987).

The next stage in assessing the quality of a plant is the probing and tasting of inner plant components, which are carried out mainly by gustatory papillae in the epipharyngeal organ (Wensler and Filshie 1969; McLean and Kinsey 1985). Once the aphid reaches its final feeding site, normally the phloem (Pollard 1973), and provided it does not encounter toxic compounds in it (Molyneux et al. 1990) it will reproduce, its performance depending on the nutritional value of the sap, as reflected in its amino acid content (Wiktelius et al. 1990) and composition (Weibull 1988).

The relative importance of visual, olfactory and gustatory stimuli in host finding is related to the feeding habit of the aphid, and the particular morph under consideration. Thus, in polyphagous morphs, such as gynoparae of *Myzus persicae* (Sulzer) and *Aphis fabae* (Scopoli), and alate virginoparae of *Rhopalosiphum padi* (L.), visual stimuli play an important role (Hodgson and Elbakhiet, 1985). In oligophagous morphs, such alatae of *Aphis gossypii* (Glover) (Pospisil, 1972) and *Cavariella aegopodii* (Scopoli) (Chapman et al., 1981), apterous virginoparae of *Cryptomyzus korschelti* (Börner) (Visser and Taanman, 1987) and alate virginoparae of *A. fabae*, and in monophagous morphs, such as gynoparae and males of *R. padi* (Pettersson 1970; Leather 1986), olfactory stimuli seem to prevail.

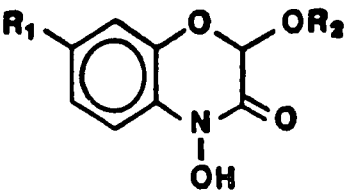
Plant secondary metabolites listed in Table 1 have been reported to be involved in aphid-plant interactions. The table includes volatiles or compounds giving rise to volatiles attracting aphids at close range, such as cyanogenic glucosides, and volatiles repelling them, such as E-β-farnesene; components of the plant wax layer which exert positive stimuli towards aphids, such as alkanes, or those which provoke negative stimuli, such as diketones; components in trichomes deleterious to aphids, such as 2-tridecanone; compounds in the mesophyll which stimulate colonization by aphids, such as certain phenolics, and those which deter them, such as hydroxamic acids; and finally phloem constituents which, depending on the specific aphid-plant combination, may act as positive or negative stimuli towards colonization by aphids, such as quinolizidine alkaloids.

These compounds, when they constitute a repellent stimulus or a source of toxicity, may in principle be manipulated by plant breeders to generate plants with enhanced barriers to aphid colonization. For this to be possible, not only the involvement of a given secondary metabolite must be determined, but an understanding of the interaction at the molecular level must be developed, and suitable parental germplasm for breeding must be encountered. If a modern bio-engineering approach is to be undertaken, knowledge of the genes involved in the accumulation (biosynthesis vs. degradation) of the compound and the associated gene physiology, must be secured.

One of the best described examples of plant secondary compounds involved in plant-aphid interactions are hydroxamic acids (Hx) (Fig. 2). They are a family of compounds of the 4-hydroxy-1,4-benzoxazin-3-one type present in the Gramineae, which show good promise as a defense mechanism in cereals (Niemeyer 1988).

Table 1. Principal classes of plant secondary compounds involved in aphid-host interactions

COMPOUNDS	PLANT	APHID	REFERENCE
Cyanogenic glucosides	*Prunus*	*R. padi* 1986	Petterson, 1973; Leather, 1986
Glucosinolates	*Brassica*	*Brevicoryne brassicae* (L.) *M. persicae* *Lipaphis erysimi* (Kaltenbach)	Pettersson, 1973; Wensler, 1962 Weber *et al.*, 1986 Gill and Bakhetia, 1985
"Host plant odours"	*Stachis* *Cucurbita*, *Thunbergia*	*C. korschelti* *A. gossypii*	Visser and Taanman, 1987 Pospisil, 1972
Alkanes	*Vicia*	*Acyrtosiphom pisum* (Harris)	Klingauf, 1978
Diketones	*Triticum*	*S. avenae*	Lowe *et al.*,1985
Phenols	*Vicia*, *Beta*	*A. fabae*	Jordens-Rittger, 1979
Phlorizin	*Malus*	*Aphis pomi* (DeGeer)	Montgomery and Arn, 1974
Alkaloids	*Nicotiana*	*R. maidis*	Abernathy and Thurston, 1969
2-Tridecanone	*Lycopersicon*	*A. gossypii*	Williams *et al.*, 1980
E-β–Farnesene	*Solanum*	*M. persicae*	Lapointe & Tingey, 1986; Avé *et al.*, 1987
Quinolizidine alkaloids	*Sarathamnus* *Lupinus* *Petteria* *Cytisus* *Lupinus*	*Acyrtosiphon spartii* (Koch) *A. pisum* *Aphis cytisorum* (Hartig) *Macrosiphon albifrons* = (Harris)	Smith, 1966 Wegorek and Krzymanska, 1971 Wink and Witte, 1985 Wink and Romer, 1986
Indol alkaloids	*Hordeum*	*S. graminum*, *R. padi*	Zúñiga and Corcuera, 1988
Phenolics	*Malus* *Rosa* *Populus*	*Eriosoma lanigerum* (Hausmann) *Macrosiphum rosae*(L.) *Pemphigus betae* (Doane)	Sen Gupta and Miles, 1975 (Hausmann) Miles, 1985 Zucker, 1982
Coumarins	*Vicia*	*Aphis craccivora* (Koch)	Mansour et al., 1982
Cardiac glycosides	*Asclepiadaceae*	*Aphis nerii* (Boyer)	Rothschild et al., 1970
Hydroxamic acids	*Gramineae*	*M. dirhodum*, *S. graminum* *S. avenae*, *R. padi*, *R. maidis*	Niemeyer, 1988; Leszczynski *et al.*, 1989



R_1 = H, CH_3O
R_2 = H, glucosyl
DIMBOA: $R_1 = CH_3O$, $R_2 = H$
DIBOA: $R_1 = H$, $R_2 = H$

Fig. 2. Main hydroxamic acids in the Graminese.

Hydroxamic Acids in Cereal Resistance to Aphids

Hydroxamic acids (Hx) were discovered over three decades ago in relation to fungal diseases of rye (Virtanen and Hietala, 1960). Later found in maize, they were associated with resistance to the European corn borer, *Ostrinia nubilalis* (Hübner). Breeding programs led to the production of maize cultivars with high Hx levels which were resistant to leaf feeding by the first brood of the borer (Grombacher et al. 1986; Guthrie et al. 1986).

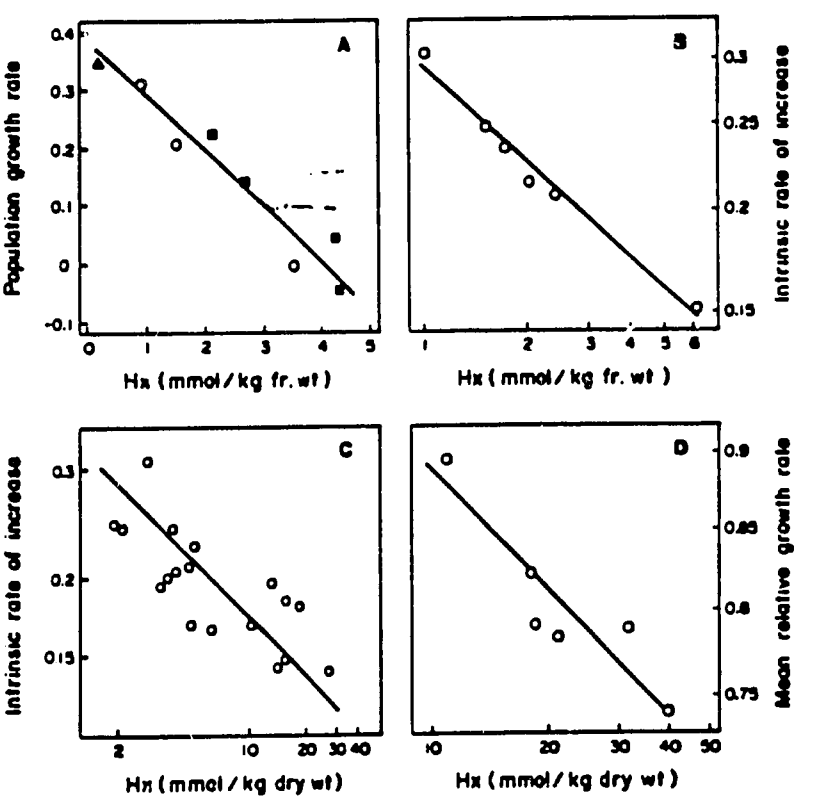


Fig. 3. Hydroxamic acid in plants and aphid population increase measured as population growth rate [A, ▲ = barley, ○ = wheat, ■ = rye] (Argandoña et al., 1980), intrinsic rate of natural increase [B and C] (Bohidar et al., 1986; Thackray et al., 1990a), or mean relative growth rate [D] (Thackray *et al.*, 1990b).

79

The first work relating Hx with aphid performance refers to *Rhopalosiphum maidis* (Fitch) growing on different maize inbreds (Long et al. 1977). Inverse correlations occur between populations of aphids accumulated on plants and Hx levels in the plants, both in greenhouse and field experiments (Beck et al. 1983). Further studies on aphid performance on cultivated and wild Gramineae in relation to Hx levels during the past dozen years have further substantiated the correlations described. Figure 3 summarizes the data for *Metopolophium dirhodum* (Walker), *Sitobion avenae* (F.), *Schizaphis graminum* (Rondani) and *R. padi*, in which aphid performance is determined in three different ways: population growth rate (Argandoña et al. 1980; Corcuera et al. 1982), intrinsic rate of natural increase (Bohidar *et al.* 1986; Thackray et al., 1990a) and mean relative growth rate (Thackray et al. 1990b). Two of these correlations individually include widely different taxa, e.g. Figure 3A includes barley, rye and wheat, and Figure 3B includes tetrapolid and hexaploid wheats: *Triticum durum*, *T. polonicum*, *T. dicoccum* and *T. aestivum*.

Incorporation of DIMBOA into excised barley leaves originally lacking Hx, and measurement of the performance of *M. dirhodum* on them, leads to a similar linear correlation (Argandoña *et al.* 1980). Moreover, survival of *R. maidis*, *S. graminum* and *M. dirhodum* decreases with increasing DIMBOA concentrations in artificial diets where aphids feed from (Corcuera et al. 1982), lending credibility to the interpretation that Hx are the causative factor in the correlations of Figure 3, and pointing to an antibiotic effect of Hx on aphids.

When *S. graminum* is allowed to feed for a short period of time from artificial diets containing different DIMBOA concentrations, and is then transferred to control diet without DIMBOA, mortality first increases and then decreases with increasing DIMBOA concentrations in the original diet (Argandoña et al. 1983). Additionally, the concentrations of DIMBOA found in *S. avenae* which had been feeding on artificial diets or wheat plants with increasing DIMBOA levels go through a similar maximum (Niemeyer et al. 1989).

Electronic monitoring of the feeding behavior of *S. graminum* in artificial diets containing different DIMBOA levels shows that the average ingestion period is inversely correlated with DIMBOA levels (Argandoña et al. 1983).

In dual choice tests between wheat cultivars differing in DIMBOA levels, *R. padi* shows a significant tendency to preferentially settle on those plants with lower DIMBOA levels. Electrical penetration graphs (Tjallingii 1990) showed that in wheat seedlings with higher DIMBOA levels fewer aphids reached the phloem within a given time, and they needed longer times to contact a phloem vessel than in those with lower levels (Figure 4) (Givovich and Niemeyer 1990). These experiments demonstrate a feeding deterrent effect of DIMBOA on aphids before the aphid reaches the phloem. Moreover, the time an aphid remains feeding in the phloem is independent of the DIMBOA level

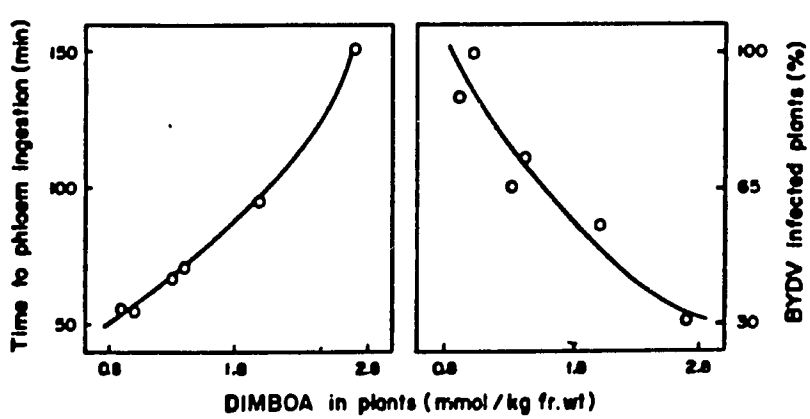


Fig. 4. Ability of *Rhopalosiphum padi* to reach the phloem and to infect with BYDV, plants of wheat cultivars with different levels of hydroxamic acids.

determined in the seedling. The antibiotic effect of DIMBOA described for aphids feeding on wheat plants, may be due to the effect of DIMBOA ingested prior to reaching the phloem or to levels in the phloem which result in antibiosis but are insufficient to provoke feeding deterrency.

Feeding deterrency by DIMBOA opens the possibility of these compounds bearing on transmission of barley yellow dwarf virus (BYDV) by aphids. When nymphs of *R. padi* carrying BYDV feed on seedlings of wheat cultivars with different DIMBOA levels, fewer seedlings are infected with BYDV in the high DIMBOA cultivars than in the low ones (Figure 4). In agreement with this finding, in a survey of 17 wheat lines in current use in Chile, BYDV tolerance was found in the six highest DIMBOA lines and BYDV susceptibility was found in the next nine lower DIMBOA lines. The two lines with the lowest DIMBOA levels were classified as tolerant, possibly reflecting some virus-host interaction preventing virus from establishing or replicating effectively (Givovich and Niemeyer 1990).

The search for conditions for the safe manipulation of Hx and for a molecular interpretation of its biological activities has led to detailed studies of their chemistry. The basis for the reactivity of DIMBOA is provided by the fast equilibration in solution of the hemiacetal (predominant) with an aldol. These molecules show organic functions which are highly reactive towards nucleophiles (Figure 5). Thus, thiols reduce the nitrogen atom producing a lactam and form hemithioacetals by addition to the aldehyde group (Atkinson et al. 1990), and amines form Schiff bases with both the aldehydic and the hydroxamic carbonyl groups (Pérez and Niemeyer 1989b). Furthermore, papain, a proteolytic enzyme with a highly reactive cysteine at the active site, is inactivated by DIMBOA by reaction of the hydroxamic nitrogen atom with the reactive sulphydryl group (Pérez & Niemeyer 1989a), and α-chymotrypsin, a proteolytic enzyme with a hyper reactive serine at the active site, is inactivated by DIMBOA by reaction of the hydroxyl group of this serine, and possibly the amino group of lysine residues, with the hemiacetal (or aldol) (Cuevas et al. 1990).

These studies with model organic compounds and enzymes constitute the basis for the interpretation of the effect of DIMBOA on aphid enzymes. Acetylcholinesterases in insects are key enzymes in the transmission of nerve impulses and are the targets of organophosphorous and carbamate insecticides (Hoover 1980). Acetylcholinesterase has an essential serine at the active site. In addition, aphid cholinesterases have a catalytically important cysteine residue elsewhere (Zahavi et al. 1972; Manulis et al. 1981; Brestkin et al. 1985).

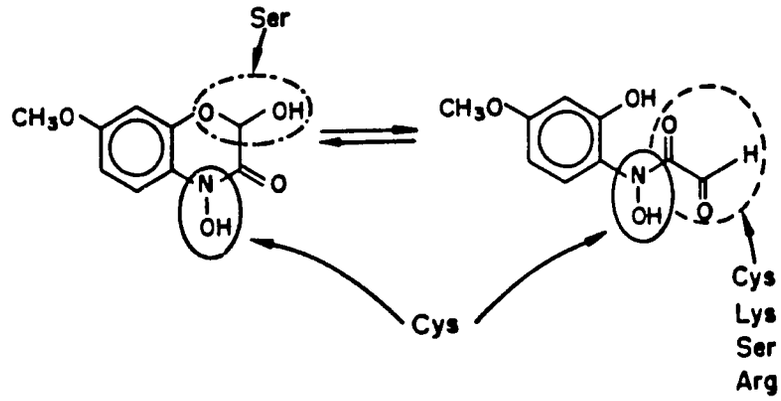


Fig. 5. Organic functions responsible for the reactivity of hydroxamic acids in solution. Abbreviations refer to aminoacid residues from enzymes.

Acetylcholinesterases isolated from *R. padi* show a pH optimum of 7 and are inhibited by thiol reagents, similar to results with acetylcholinesterases of other aphids. They are inactivated by DIMBOA, presumably by reaction of cysteine and serine residues with the nitrogen atom and the hemiacetal function. This inactivation may account for the antibiotic effects of DIMBOA on aphids (Cuevas and Niemeyer 1990).

Surveys of Hx levels in different taxa of the Gramineae (Zúñiga et al. 1983; Niemeyer 1988b; Thackray et al. 1990a) reveal variations of Hx levels in cultivated wheats ranging from 1 to 10 mmol/kg fr. wt at the age of highest accumulation in the seedling (Argandoña et al. 1981), and availability of wild grasses with unusually high levels of Hx, e.g. nearly 40 mmol DIMBOA/kg fr. wt in wild *Secale* species and 12 and 16 mmol DIBOA/kg fr. wt in wild *Critesion* and *Elymus* species, respectively (Copaja et al. 1990).

Additionally, DIBOA appears in all wild species examined in the genus Hordeum, but is absent from barley cultivars studied (Barría et al. 1990). The results provide a rationalization of earlier results on performance of *R. padi* in wild and cultivated barley (Weibull 1987).

It seems desirable to increase the content of Hx in agronomically interesting wheats. Genetic engineering appears as a valid alternative, provided that further knowledge is gained on the enzymology and molecular biology of these metabolites (Bailey and Larson 1989).

Given the very sophisticated way in which cereal aphids feed (Pollard 1973; Tjallingii 1990), it is essential to examine the tissue and sub-cellular localization of hydroxamic acids.

Hydroxamic acids are present in the intact plants as glucosides which are broken down to the active aglucones by endo β-glucosidases when the tissue is injured (Hofman and Hofmanová 1969). Since aglucones are also phytotoxic (Queirolo et al. 1983), it is expected that the hydrolytic enzymes are present in a compartment different from that containing the glucosides. It is desirable to study tissue and sub-cellular localization of β-glucosidases and to determine in which form these metabolites are ingested by aphids.

Although biological control and host plant resistance are often compatible and even complementary (Starks et al. 1972; Pimentel and Wheeler 1973; Schuster et al. 1976; van Emden and Wratten, this volume), it has been demonstrated that variation in host insects, in particular through variations in their host plants, may have dramatic effects on their predators and parasitoids (Salt 1941; Vinson 1976; Powell and Zhi-Li 1983; Budenberg and Powell 1988). These effects may even render incompatible efforts to increase plant antibiosis with other pest control management practices (Bergman and Tingey 1979; Campbell and Duffey 1979; Kennedy 1974). It would be desirable to examine the performance of aphid predators and parasitoids when they interact with aphids settled on plants containing different Hx levels.

Conclusions

Knowledge about the interaction of aphids with plants, in particular those in which aphids constitute a pest of crop plants, has grown mostly in the descriptive, phenomenological aspects. Only lately has the intimate nature of the interactions begun to be explored. The days seem not so distant when a rational, predictable, bio-engineering approach to crop improvement will be possible by enhancement of natural plant defenses.

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87

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HYDROXAMIC ACID CONTENT OF PERENNIAL TRITICEAE

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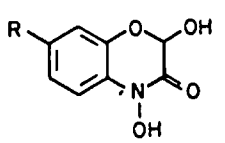
Key Word Index—Gramineae; Triticeae; wheat breeding; hydroxamic acids; DIMBOA; DIBOA.

Abstract—Ninety-four accessions of perennial Triticeae were examined for content of the hydroxamic acids DIBOA and DIMBOA. Levels of DIBOA (up to 44 mmol kg^{-1} fr. wt) were higher than those of DIMBOA (up to 4.5 mmol kg^{-1} fr.wt). The genera *Hordeum*, *Psathyrostachys* and *Secale* did not contain DIMBOA. Some accessions represented potentially useful germplasm for producing wheat with high levels of hydroxamic acids through wide hybridization.

INTRODUCTION

Hydroxamic acids (Hx) derived from 4-hydroxy-1,4-benzoxazin-3-one have been isolated from extracts of cereals such as wheat, maize and rye [1]. The main Hx in wheat and maize is 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (**1**) (DIMBOA) [2, 3] while in rye it is the demethoxylated analogue (**2**) DIBOA [4]. This family of compounds has been associated with resistance of plants to bacteria [5], fungi [6] and insects. Among the best studied cases of Hx involvement in insect resistance, are maize resistance to the European corn borer, *Ostrinia nubilalis* [7] and wheat resistance to cereal aphids [8, 9]. Hydroxamic acids are deleterious to aphids through antibiosis [10, 11] and antixenosis [12]. This latter property has been invoked in the decrease of aphid transmission of barley yellow dwarf virus to wheat plants containing high levels of Hx [13]. Hydroxamic acids play also an important role in detoxification of atrazine-derived herbicides [14] and in allelopathic effects in cereals [15, 16].



1 DIMBOA : R = OMe
2 DIBOA : R = H

Breeding for high Hx levels in maize has been suggested in order to obtain resistance towards leaf feeding by the first brood of the European corn borer [17–20]. The inheritance of Hx in maize has been studied [21] and suitable germplasm for breeding has been described [17]. Potential parental material for breeding wheat for high Hx levels has been screened in accessions of the genus *Triticum* [22], and in other genera of the Gramineae [2]. We report here on DIBOA and DIMBOA levels in seedlings of perennial Triticeae.

RESULTS AND DISCUSSION

The DIBOA and DIMBOA levels in accessions from 10 genera of the Triticeae containing mostly perennial species are given in Table 1. Classification and naming of the Triticeae is under debate [23–27]; the genomic system proposed by Dewey [23] has been used in this work.

Hx levels vary considerably with plant age: Hx begin to accumulate upon germination of the seed, which lacks Hx [10], reaching a maximum level after a few days and then decreasing [2, 8, 10]. In order to produce comparable results, the levels of DIBOA and DIMBOA were followed in all accessions for 15 days after germination. Within this period, Hx levels reached a maximum, which occurred between 4 and 8 days, depending upon the accession studied. Values reported in Table 1 represent Hx levels at the plant age where the maximum occurred.

Hx levels have also been shown to be influenced by growing conditions such as light intensity [28], photoperiod [29], temperature [30] and mineral and water availability [31]. Seedlings in the experiments described were subjected to comparable external conditions. The sensitivity of different accessions to particular environmental conditions may, however, be different.

Different accessions showed considerable differences in the proportion of water in the tissue. The results are presented in terms of fresh weight, as this variable is best related to the concentration to which a pest may be exposed. It should be borne in mind, however, that compartmentation of Hx within the plant may influence the relationship between the plant and the attacking organism.

There is substantial variation among perennial Triticeae in DIBOA and DIMBOA levels (Table 1). With only a few exceptions, levels of DIBOA were higher than of DIMBOA, particularly in *Critesion*, *Elymus* and *Pseudoroegneria*. Moreover, DIMBOA was not detected in any of the accessions examined in the genera *Hordeum*, *Secale* and *Psathyrostachys*. Only in *Agropyron*, *Thinopyrum* and *Elytrigia* were DIBOA and DIMBOA levels similar.

Agropyron (genome P), *Pseudoroegneria* (genome S) and *Elytrigia* (genome SX) show low levels of both

Table 1. Hydroxamic acid content in the perennial genera of the Triticeae

Genus	Species	Number of accessions studied	Hydroxamic acids (mmol kg^{-1} fr.wt) Concentration range DIBOA	DIMBOA	Mean concentration for genus DIBOA	DIMBOA
Agropyron	*cristatum*	9	1.27–0.08	0.77–0.05	0.40	0.30
	c. puberulum	1	N. D.	0.17		
	desertorum	3	0.20–0.17	0.37–0.07		
	fragile	2	0.50–0.12	0.26–0.05		
Critesion	*bogdanii*	3	6.68–0.22	0.43–ND	3.46	0.37
	brevisubulatum	4	11.56–2.80	0.48–ND		
	b. iranicum	4	5.80–1.45	1.04–ND		
	b. violaceum	1	1.56	0.13		
	californicum	2	2.21–1.66	0.51–0.22		
	capense	1	2.17	0.56		
	chilense	2	5.25–1.25	0.40–0.19		
	lecheri	1	6.90	0.05		
	stenostachys	1	1.26	0.42		
Elymus	*alataricus*	1	5.41	1.33	5.71	0.67
	canadensis	1	16.96	ND		
	dahuricus	1	ND	0.12		
	drobovii	1	2.87	1.15		
	elymoides	1	4.88	0.80		
	lanceolatus	2	6.41–2.41	0.20–ND		
	mutabilis	1	5.56	ND		
	patagonicus	1	9.24	0.09		
	scabriglumis	1	3.37	ND		
Elytrigia	*elongatiformis*	2	0.16–0.071	0.25–0.26	0.11	0.26
Hordeum	*bulbosum*	1	1.40	ND	1.25	ND
	depresum	1	0.82	ND		
	intercedum	1	1.20	ND		
	murinum	1	0.23	ND		
	procerum	1	2.58	ND		
Leymus	*arenarius*	1	8.61	0.53	2.86	1.22
	angustus	2	2.08–1.23	3.05–0.70		
	cinereus	2	9.90–4.66	0.41–ND		
	karelinii	2	1.60–0.44	4.47–1.0		
	multicaulis	1	2.01	0.87		
	racemosus	2	2.69–0.64	0.87–1.27		
	triticoides	1	0.44	0.89		
Secale	*montanum*	1	31.66	ND	31.14	ND
	m. chaldicum	1	18.08	ND		
	m. kuprijanovi	1	43.67	ND		
Thinopyrum	*caespitosum*	2	1.20–0.71	1.22–0.40	0.70	1.23
	intermedum	3	2.67–0.82	2.38–1.72		
	junceum	2	0.16–0.07	0.81–0.35		
	nodosum	1	0.55	0.21		
	podperae	2	0.66–0.28	1.58–1.40		
	ponticum	2	0.11–0.07	2.84–1.87		
	p. turcicum	1	0.29	0.44		
Pascopyrum	*smithii*	3	0.18–0.09	1.53–0.79	0.14	1.13
Psathyrostachys	*juncea*	1	9.54	ND	14.45	ND
	j. bozoisky	1	12.99	ND		
	j. vinall	1	10.67	ND		
	fragilis	1	24.59	ND		
Pseudoroegneria	*libanotica*	4	1.53–0.57	0.36–0.05	0.93	0.26
	spicata	2	0.88–0.76	ND		
	spicata-inermis	1	1.24	ND		
	stipifolia	3	1.20–0.28	0.37–ND		

hydroxamic acids. These three genera have traditionally been grouped together under *Agropyron* [23, 27]. Also showing low levels of both acids is *Thinopyrum* (genome J). This genus has been placed together with *Pseudoroegneria* and *Elytrigia* in the conventional treatment of Tzvelev [32].

Psathyrostachys (genome N) is taxonomically one of the least controversial genera in the Triticeae and has only DIBOA. *Critesion* (genome H) on the other hand, is genomically heterogeneous and has been the subject of long controversy. Traditionally it has been a part of *Hordeum* (genome I). The two genera can, however, be separated on the basis of hydroxamic acid levels: while *Hordeum* contained only DIBOA and in relatively low levels, *Critesion* contained both acids, DIBOA being present in higher concentration and, in some accessions, in rather high absolute levels.

Analysis of *Leymus* (genome JN) and *Elymus* (genome SHY) showed the presence of both acids, with DIBOA predominating over DIMBOA in both genera. Interestingly, *Leymus* is part of *Elymus* in traditional treatments of the Triticeae [33].

The genus *Secale* contains a single perennial species, *S. montanum*. It contained only DIBOA, in unusually high levels. Analysis of cultivated rye also included a cultivar with unusually high level of DIBOA [34].

The genetic variability of cultivated wheats has suffered a continuous decrease in recent years [35]. Genetic diversity is a necessary prerequisite for the production of cultivars with pest and disease resistance, wide adaptation and increased yields. The restoration of variability in the genetic material of cultivated wheats may be accomplished by exploiting the genetic resources of some of its wild relatives.

Crosses have been produced between wheat and all the perennial genera of the Triticeae, with the exception of *Psathyrostachys*, thus permitting the transfer of useful characters [25, 36–39]. The results in Table 1 show that high levels of hydroxamic acids may also be transferred into wheat through wide hybridization with the perennial Triticeae.

EXPERIMENTAL

Plants. Seeds were grown under a 12L:12D photoperiod at 22° with a 3° range.

Reference compounds. DIMBOA was isolated from ethereal extracts of *Zea mays* cv T129, as described previously [40]. DIBOA was synthesized essentially as described in ref. [41].

Analytical method. Aerial parts of the seedlings (20–50 mg fr. wt) were macerated successively with 3 × 0.33 ml H_2O, using a mortar and pestle. The aq. extract was left at room temp. for 15 min, taken to pH 3 with 0.1 N H_3PO_4 and centrifuged at 10000 rpm for 10 min. Aliquots of the supernatant (50 μl) were filtered (0.45 mμ) and then injected into a high performance liquid chromatograph. An RP-100 Lichrosfer-C18 column was used with a constant solvent flow of 1.5 ml min^{-1} and the following linear gradients between solvents A (MeOH) and B (0.5 ml H_3PO_4 in 1 l H_2O): 0–9.5 min, 30–50% A; 9.5–10 min, 50–30% A; 10–13 min, constant at 30% A. Detection was carried out at 263 nm. R_ts were 4.5 ± 0.3 min for DIMBOA and 3.5 ± 0.3 min for DIBOA.

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Hydroxamic acid levels in Chilean and British wheat seedlings

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Summary

Fifty-two cultivars of Chilean wheats were screened, using high pressure liquid chromatography, for hydroxamic acid levels at the seedling stage when maximal levels occur. DIMBOA levels ranged from 1.4 to 10.9 mmol/kg fr. wt and DIBOA levels ranged from 0 to 1.1 mmol/kg fr. wt. The family of wheat cultivars leading through breeding to the British cultivar Maris Freeman was examined. While DIMBOA and DIBOA levels in the ancestors ranged from 1.6 to 3.8 mmol/kg fr. wt and 0.16 to 0.34 mmol/kg fr. wt, respectively, DIMBOA and DIBOA levels in Maris Freeman were 2.39 and 0.24 mmol/kg fr. wt, respectively. The results are discussed in terms of the possibility of breeding for aphid resistance by making use of hydroxamic acids.

Key words: Hydroxamic acids, DIMBOA, DIBOA, wheat, aphid resistance, wheat breeding

Introduction

Hydroxamic acids (Hx) of the 4-hydroxy-1,4-benzoxazin-3-one type present in extracts of cereals such as wheat, maize and rye (Niemeyer, 1988) have been related to resistance of the plant to aphids (Long, Dunn, Bowman & Routley, 1977; Argandoña, Luza, Niemeyer & Corcuera, 1980; Argandoña, Niemeyer & Corcuera, 1981; Bohidar, Wratten & Niemeyer, 1986; Thackray, Wratten, Edwards & Niemeyer, 1990*a,b*; Wratten, Martin, Rhind & Niemeyer, 1990), components of resistance being both antibiosis (Corcuera, Argandoña & Niemeyer, 1982) and antixenosis (Argandoña, Corcuera, Niemeyer & Campbell, 1983; Givovich & Niemeyer, 1990; Leszczynski & Dixon, 1990; Leszczynski, Wright & Bakowski, 1990). The main Hx in wheat extracts is DIMBOA while in rye it is the demethoxylated analogue DIBOA (Fig. 1). Both compounds show comparable toxicity towards the greenbug *Schizaphis graminum* (Rond.) (Zúñiga, Argandoña, Niemeyer & Corcuera, 1983).

Potential limitations of hydroxamic acids in breeding programs for developing aphid resistant cereal cultivars have been pointed out: i) negative correlations between aphid performance and Hx levels are strong when *Triticum aestivum* (L.) and *T. durum* (L.) wheats are examined, but inclusion of primitive diploid and tetraploid wheats decreases considerably the correlation coefficient (Thackray *et al.*, 1990*a*); ii) after a steep increase of Hx levels soon after germination, levels drop in the plant as a whole, casting doubt on Hx as protective compounds against pests of mature plants (Argandoña *et al.*, 1981; Thackray *et al.*, 1990*a*); and iii) if correlations between resistance and Hx levels hold true only for modern wheats, germplasm available for breeders to increase Hx levels in wheat may be thought to be considerably limited.

This paper shows that within a limited set of modern wheats considerable variation of Hx levels may be found. The arguments raised above are also discussed.

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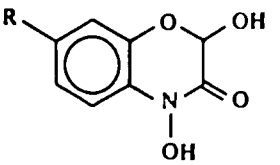
91



DIBOA: R = H
DIMBOA: R = CH_3O

Fig. 1. Structures of hydroxamic acids from cereals.

Materials and Methods

Plant material

Seeds of Chilean cultivars of *T. durum* and *T. aestivum* were obtained from Instituto de Investigaciones Agropecuarias (INIA), Chile, and seeds from British cultivars were obtained from Plant Breeding International, Cambridge, UK. They were planted in 7-cm diameter, polystyrene pots containing vermiculite and were germinated in a growth chamber at 25 °C with a 6 °C range, with a 12 light: 12 dark photoperiod and 55% humidity with a 10% range. Light intensity was 75 $\mu E\ m^{-2}\ s^{-1}$. Hx levels were determined 4, 5, 7, 8, 10 and 15 days after germination.

Reference compounds

DIMBOA was isolated from extracts of *Zea mays* (L.) cv. T129, as described by Queirolo, Andreo, Niemeyer & Corcuera (1984). DIBOA was synthesised as described by Jernow & Rosen (1975).

Hydroxamic acid quantification

Aerial parts of seedlings (20 – 70 mg fr. wt, usually equivalent to 3 to 4 seedlings) were macerated successively with three batches of 0.33 ml H_2O each, using a mortar and pestle. The aqueous extract was left at room temperature for 15 min and was then taken to pH 3 with 0.1 NH_3PO_4. The extract was then centrifuged at 10 000 g for 10 min and the supernatant filtered (0.45 mμ). Fifty μl of the filtrate were injected into a high performance liquid chromatograph (Shimadzu LC-6A). A Lichrosfer RP-18 column (100 × 4 mm) was used with a constant solvent flow of 1.5 ml/min and the following linear gradients between solvents A (MeOH) and B (0.5 ml H_3PO_4 in 1 litre H_2O): 0 to 9.5 min, 30 to 50% A; 9.5 to 10 min, 50 to 30% A; 10 to 13 min, constant at 30% A. Detection was carried out at 263 nm. Retention times were 3.5 ± 0.3 min for DIMBOA and 4.5 ± 0.3 min for DIBOA.

Three replicated analyses were carried out for each Chilean cultivar and eight for each British cultivar.

Results and Discussion

Hydroxamic acid levels of a broad spectrum of currently used Chilean cultivars are shown in Fig. 2. Levels reported correspond to the age at which seedlings contained maximal levels, usually around the fourth or fifth day after germination. While at this stage of development some cultivars showed measurable levels of DIBOA, these levels decreased and became unmeasurable in all cases after the tenth day, probably reflecting the transformation of DIBOA into DIMBOA; this has also been observed in wheat callus culture (Zúñiga, Copaja, Bravo & Argandoña, 1990).

CV

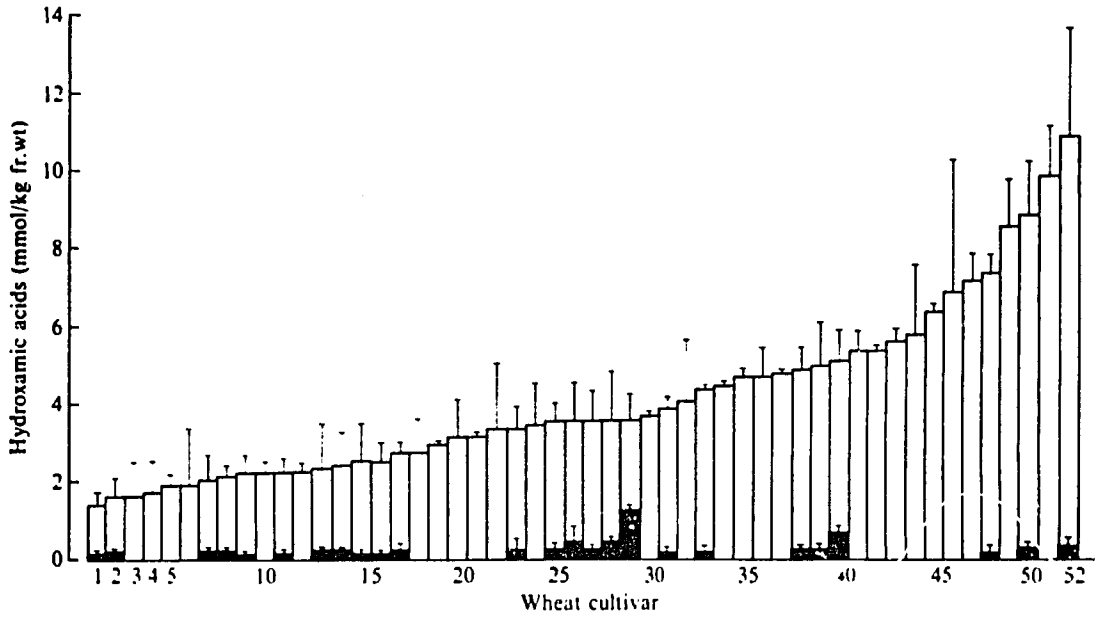


Fig. 2. Maximum DIBOA (■) and DIMBOA (□) levels in seedlings of Chilean wheat cultivars: 1 (Perquenco), 2 (Vilufén), 3 (Antufén), 4 (Andalién), 5 (Tahuén), 6 (Lilén), 7 (Nobo), 8 (Huenufén), 9 (Canelo), 10 (Reihue), 11 (Lancero), 12 (Ancoa), 13 (Pumafén), 14 (Maitén), 15 (Chifén), 16 (Talafén), 17 (Rancofén), 18 (Laurel), 19 (T-1500), 20 (Aurifén), 21 (Malifén), 22 (Yafén), 23 (Budifén), 24 (Mexifén), 25 (Lautaro), 26 (Collafén), 27 (Cunco), 28 (Patagua), 29 (Labriego), 30 (Etoile de Choisy), 31 (Millaleu), 32 (Linaza), 33 (Manella), 34 (Likafén), 35 (Manquefén), 36 (Vilmorin), [illegible] (Cisne), 38 (Toquifén), 39 (Chasqui), 40 (Lanco), 41, (Panguifén), 42 (Lilifén), 43 (Lancofén), 44 (Sonka-INIA), 45 (Andifén), 46 (Trisa), 47 (SNA-3), 48 (Naofén), 49 (Likay), 50 (Sipa), 51 (Alifén) and 52 (Quilafén). All cultivars were *T. aestivum*, except for 47 and 52, which were *T. durum*. Bars represent the upper 95% confidence limit.

The levels of DIMBOA ranged from 1.4 to 10.8 mmol/kg fr. wt. In artificial diets, DIMBOA has been shown to significantly decrease aphid reproduction rates at concentrations as low as 0.05 mM (Corcuera *et al.*, 1982), corresponding approximately to a bulk tissue concentration of 0.05 mmol/kg fr. wt. Hence, even in those cultivars containing the lowest levels of DIMBOA, deleterious effects may be expected on aphid pests attacking the plant at this stage of development, provided the overall DIMBOA concentrations reflect that encountered by the feeding aphid. Although the precise distribution of Hx within a plant remains unknown, they are mainly concentrated in the vascular bundles (Argandoña & Corcuera, 1985; Argandoña, Zúñiga & Corcuera, 1987). [illegible] site of aphid feeding (Pollard, 1973).

Wild diploid and tetraploid wheats do not follow the pattern of aphid performance in relation to Hx levels followed by modern tetraploid and hexaploid wheats (Thackray *et al.*, 1990*a*). This is not unexpected since different taxa, especially relatively distant ones, may depend on different combinations of mechanisms for their defence against a given pest. On the other hand, large variations may be expected between individual plants when measuring quantitative traits in wild populations. Hence, the different patterns found when these plants are analysed may also be caused partly by aphid performance having, of necessity, been measured on individual plants different from those used in assessing Hx levels, coupled with the fact that the number of replications performed may have been too low.

The decrease in Hx levels with age of the plant has been taken as an argument against Hx being a character desirable to be bred into agronomically useful wheat. It should be

93

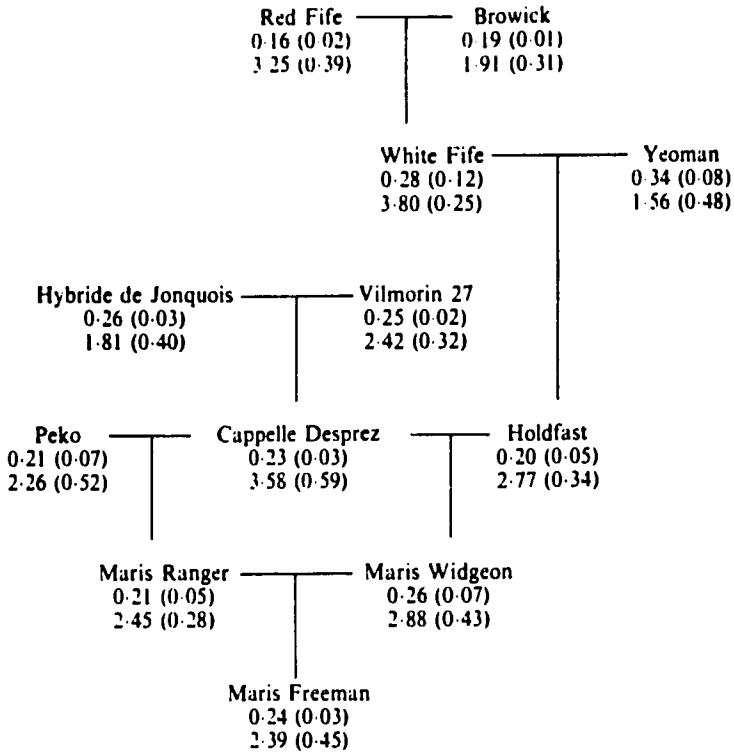


Fig. 3. Pedigree of English winter wheats. Values given are mean DIBOA (above) and DIMBOA (below) levels in seedlings, with 1.96 × S.E.

emphasised, however, that although this decrease does occur when the plant is considered as a unit, the youngest tissue in the plant still retains a high Hx level. Thus, in a 6-leaf wheat plant, while the oldest leaf may contain 0.6 mmol/kg fr. wt, the youngest may contain as much as 2.2 mmol/kg fr. wt (Thackray *et al.*, 1990*a*). The Hx concentration determined for the plant as a whole would lie closer to the value determined for the oldest leaf, since it is this one which contributes most to plant weight. Moreover, the flag leaf has been shown in a small selection of *Triticum* material to contain high Hx levels (Thackray *et al.*, 1990*a*; Leszczynski *et al.*, 1990). This pattern of distribution of Hx within the plant may be of importance in the defence of the plant against organisms colonising younger tissue, and is therefore relevant to the aphids *Sitobion avenae* (F.), which feeds on flag leaves and ears (Wratten, 1975) and *Rhopalosiphum padi* (L.), which is a seedling pest in western Europe (Vickerman & Wratten, 1979).

Fig. 3 shows Hx levels in a group of related wheats consisting of cultivars bred in the UK during the course of the present century and their immediate foreign ancestors (Lupton, 1987). The values show that the search for agronomically useful traits such as yield and disease resistance has not been paralleled by significant changes in Hx levels. Additionally, the average values fall well below the highest values determined in Chilean wheats, showing considerable potential for breeding for higher Hx levels by exploiting a wide, internationally-available range of *Triticum* material.

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91

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OCCURRENCE OF DIBOA IN WILD *HORDEUM* SPECIES AND ITS RELATION TO APHID RESISTANCE

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(*Received* 25 *April* 1991)

Key Word Index—*Hordeum*; Gramineae; aphid resistance; *Rhopalosiphum padi*, hydroxamic acids; DIBOA.

Abstract—The secondary metabolite 2,4-dihydroxyl-1,4-benzoxazin-3-one (DIBOA) was found in seedlings of wild *Hordeum* species, but not of cultivated barley (*Hordeum vulgare*). DIBOA levels correlated negatively with performance of the aphid *Rhopalosiphum padi* on seedlings.

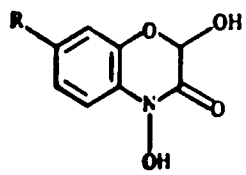
INTRODUCTION

In wheat and maize, the hydroxamic acid DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) [1] plays a major role in aphid resistance. The growth of aphids and their populations on wheat and maize plants correlates negatively with DIMBOA levels in the plants [2–7], the compound is toxic and alters feeding behaviour of aphids on artificial diets and on wheat plants [2, 4, 5, 8–11]. Hydroxamic acids have not been found in cultivated barley and aphid performance has been related to the presence of the indole alkaloid gramine [12]. We describe the finding of DIBOA (7-demethoxylated analogue of DIMBOA) in seedlings of wild barley (*Hordeum*) species, demonstrate that DIBOA is toxic to the aphid *Rhopalosiphum padi* in artificial diets, and show a correlation between DIBOA levels and performance of *R. padi* on plants of wild *Hordeum* species.

RESULTS AND DISCUSSION

Quantitation of DIBOA in Hordeum *spp.*

A survey of barley cultivars (*Hordeum vulgare* L.) in search of hydroxamic acids using the $FeCl_3$ method of analysis [13] did not yielded positive results [14] so, several sensitive methods were used to ascertain the presence of DIBOA in wild barley. The initial criterion was the coincidence of retention time on HPLC with that of a synthetic standard [15] and with a standard isolated from rye seedlings [16]. As DIBOA decomposes in aqueous solutions to benzoxazolin-2-one as the main product [17] and retention time of the latter compound can be easily distinguished from that of DIBOA on HPLC, aqueous extracts to be analysed were heated and aliquots withdrawn after different time intervals were analysed by HPLC. The peak attributed to DIBOA progressively disappeared and a peak with the retention time of synthetic BOA appeared in parallel [18].



	R
DIBOA	H
DIMBOA	MeO

Lastly, a GLC method was developed that allowed the separation of different hydroxamic acids and their corresponding benzoxazolinones. Extracts from wild barley produced, after derivatization, a peak with the same retention time as synthetic DIBOA. Quantification of DIBOA in extracts was carried out by HPLC.

DIBOA levels in wild barley and aphid performance

The role of hydroxamic acids in cereals in host plant resistance to aphids has been shown to involve antibiosis [4, 6, 7] and feeding deterrency [9–11]. Studies have been carried out mainly with maize and wheat, which have DIMBOA as the main Hx [8, 13]. DIBOA has been found to be toxic against the aphid *Schizaphis graminum*, when tested in artificial diets [8]. DIBOA is also toxic to *R. padi* when incorporated into artificial diets (Fig. 1).

A screening for resistance against *R. padi* has been carried out in *Hordeum* species [19]. DIBOA levels determined in the same accessions screened for aphid resistance correlated strongly with the published resistance ratings (Fig. 2). Thus, it is likely that in *Hordeum*, as in other Gramineae, hydroxamic acids play a role in aphid resistance, although this does not preclude the role of other factors such as amino acids [20].

Hydroxamic acids have not been found in cultivated barley. In these plants, aphid resistance appears to be related to the presence of indole alkaloids, such as gramine [12]. The absence of hydroxamic acids in cultivated barley and the corresponding loss of potential for resistance to aphids and to diseases towards which DIBOA is active [21], may reflect the loss of genetic variability and resistance mechanisms in modern crops [22].

96

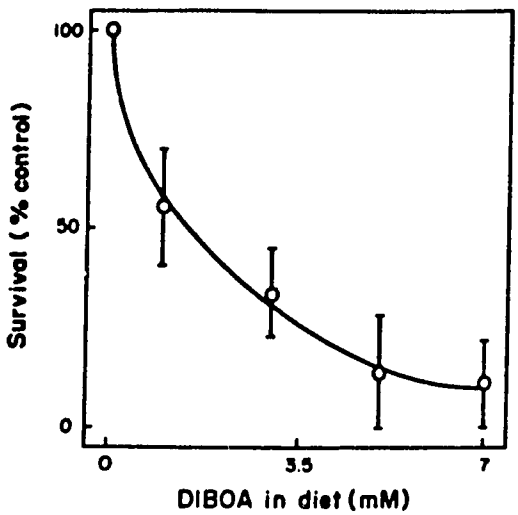


Fig. 1. Effect of DIBOA in artificial diets on survival of nymphs of *Rhopalosiphum padi*. Survival was determined after 24 hr in the minimum diet (see Experimental). For each concentration, eight samples with 10 initial aphids were followed. Mean survival of aphids fed with a diet without DIBOA was 92.5%. Vertical bars are standard errors of the mean.

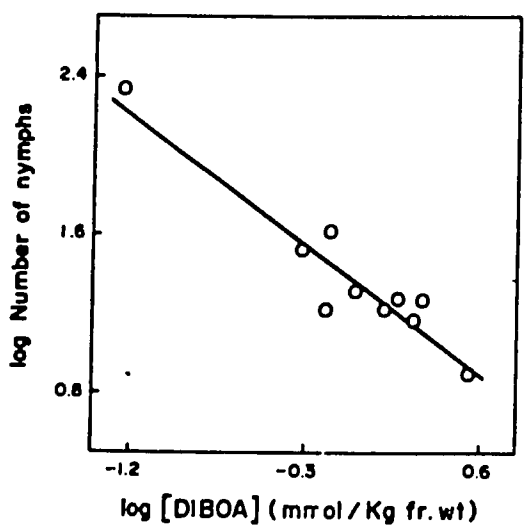


Fig. 2. Correlation of DIBOA in *Hordeum* seedlings with performance of *R. padi*. The latter variable corresponds to the number of aphids produced per new-born nymph living on a plant of a given species over a period of 16 days. Species of *Hordeum* (cultivar or accession in parentheses) used were, in order of increasing DIBOA concentrations: *vulgare* cv. Tellus, *roshevitzii* (H-179), *flexuosum* (H-1110), *lechleri* (H-1277), *jubatum* (H-2030), *lechleri* (H-1324), *brachyantherum* (H-1969), *comosum* (H-1333), *lechleri* (H-1205) and *brachyantherum* (H-1972). Aphid performance was taken from ref. [19]. The correlation coefficient for the line shown was 0.907 ($p=7$).

EXPERIMENTAL

Plants. Seedlings were grown under a 12L:12D photoperiod at 22° with a 3° range, and harvested when they were 7–8 cm tall.

Aphids. A colony originating from individuals collected in wheat fields near Santiago was kept on oat plants (*Avena sativa* L.) in a culture room with 12L:12D photoperiod and 22° with a 3° range.

Reference compounds. DIBOA and BOA were synthesized as described previously [11, 14].

HPLC analysis. Aerial tissue from seedlings (20–50 mg fr. wt) was macerated successively with 3 × 0.33 ml H_2O using a mortar and pestle. The aq. extract was left at room temp. for 15 min and then taken to pH 3 with 0.1 M H_3PO_4, centrifuged at 10000 *g* for 15 min and filtered (0.45 μm). Aliquots of the supernatant (50 μl) were examined by HPLC. The analysis was carried out with a Lichrosfer RP-18 column (100 × 4 mm) with constant solvent flow of 1.5 ml min^{-1} and the following linear gradients between solvents A (MeOH) and B (0.5 ml H_3PO_4 in 1 l, H_2O): 0–9.5 min, 30–50% A; 9.5–10 min, 50–30% A; 10–13 min, constant at 30% A. Detection was carried out at 263 nm. Retention times for DIBOA and BOA were 4.5 ± 0.3 min and 6.0 ± 0.3 min, respectively.

GC analysis. Samples were prepared in the same form as for HPLC, and were then lyophilized. Dry samples (20–50 mg) were dissolved in 50 μl of N, *O*-bis(trimethylsilyl)acetamide (BTSA) and the mixture heated at 52° for 15 min. A 5 μl sample was injected into a CBPS capillary column. The temp. gradient was 170° for 3 min followed by increases of 3° min^{-1} for 12 min. The injection port and detector were kept at 300°. The R_t for derivatized DIBOA was 9.33 ± 0.03 min.

Decomposition of DIBOA. Aq. extracts were taken to pH 8 and heated at 52°. Aliquots were taken every 2 hr and injected into the HPLC. DIBOA and BOA were quantified simultaneously.

Effect of DIBOA in artificial diet on R. padi. Aphid nymphs were withdrawn from the colony and placed on a sachet containing a full diet for 24 hr. They were then transferred to a minimum diet containing DIBOA at concentrations 0, 1, 3, 5 and 7 mM. Survival was determined after different time intervals. The full diet consisted of a mixt. of aminoacids, minerals and vitamins as described [23]. The minimum diet contained 20% sucrose in pH 6.5 Pi buffer.

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DECOMPOSITION OF 7-NITRO-2,4-DIHIDROXY-1,4-BENZOXAZIN-3-ONE IN AQUEOUS SOLUTIONS

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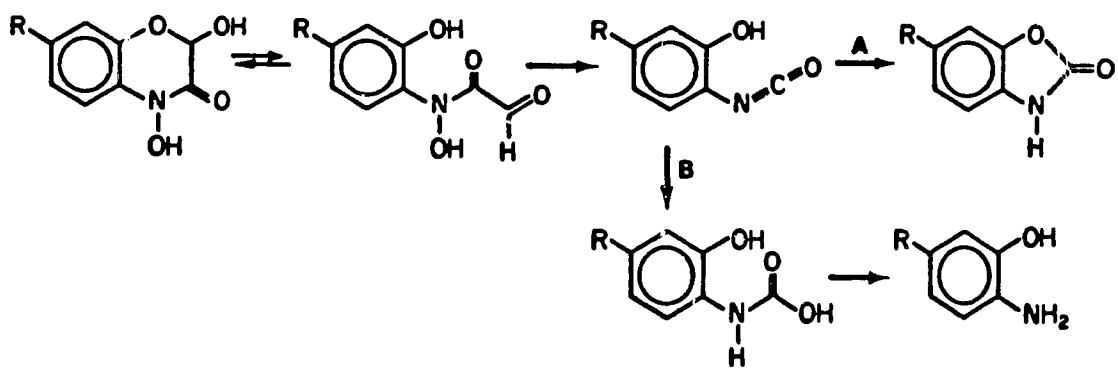
Abstract - Kinetic and product studies indicate that the title compound decomposes in aqueous solutions to give 5-nitro-2-aminophenol with the intermediacy of N-(2-hydroxy-4-nitrophenyl)-carbamic acid.

Cyclic hydroxamic acids (Hx) derived from 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) present in cereal extracts[1], show antibiotic and antifeedant effects towards cereal aphids[2-4], toxicity against several plant pathogenic fungi[5] and bacteria[6], allelopathic effects towards weeds[7-8], and possess considerable anti-inflammatory activity[9].

The decomposition of DIBOA and its 7-methoxy derivative to the corresponding benzoxazolin-2-ones have been studied in detail[10-14]. The mechanism involving an isocyanate intermediate depicted in Scheme 1 path A accounts for the observations reported. When a series of 7-substituted-DIBOAs was studied, the reactions followed a common kinetic pattern and gave rise to a Hammett plot consistent with the mechanism above[15]. The 7-nitro compound (NDIBOA) constituted an exception. We report herein the characterization of the decomposition of NDIBOA.

Scheme 1



NDIBOA was synthesized by oxidation of the corresponding lactam, which was obtained by reaction of 5-nitro-2-aminophenol with dichloroacetylchloride and further hydrolysis of the product with aqueous $NaHCO_3$[16].

The main product isolated from ethereal extracts of the decomposition reaction mixture at every pH studied was 5-nitro-2-aminophenol, identified by comparison of its spectroscopic and chromatographic properties with those of a comercial standard.

Kinetics of decomposition were followed at 50°C between pH 3 and 10 for a least 3 half-lives by taking spectra between 300 and 460 nm after different time intervals. Data was fitted to a first-order kinetic model. Standard errors of the rate constants were lower than 3%.

The spectra of reactants, intermediates and products were pH-dependent. In the pH range 3 to 7, kinetics were monophasic with disappearance of the band in the region 335-380 nm concomittant with the appearance of a band at 395 nm. Runs at pH higher than 8 showed biphasic behaviour. The first phase consisted of the decrease of the absorption in the region 390-410 nm and concomittant increase in the region 320-355 nm and showed an isosbestic point in the region 360-375 nm. During the second phase, the band in the region 320-355 nm disappeared and another one was formed in the region 360-420 nm. Kinetic plots for these processes are shown in Figure 1.

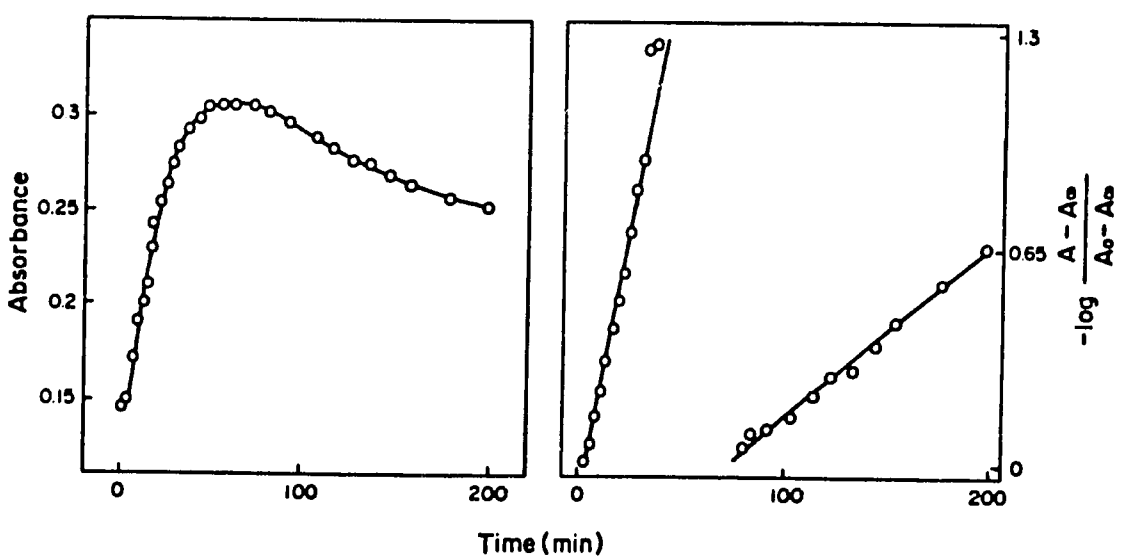


Fig. 1. Decomposition of NDIBOA at pH 10, 50°C: direct plot (A), semilog plots for the two phases (B).

Table 1 shows the rate constants obtained for the decomposition of NDIBOA at different pH. The rates for the first process (k_1) were pH-dependent. The rate-pH profile (Fig. 2) suggests the independent decomposition of NDIBOA and its monoanion. The solid

line in the graph corresponds to the least-squares fit of the data to equation 1 arising from the model in Scheme 2. The fit generates values for $k_1 = 0.94 \times 10^{-3}$ min^{-1}, $k_2 = 11.68 \times 10^{-3}$ min^{-1} and pK = 5.3. A value of 6.14 has been determined at 30°C from thermodynamic spectral data[15]. The second process (k_{II}) does not show a clear cut dependence on pH.

Table 1. pH dependence of the pseudo first order rate constants for the decomposition of NDIBOA at 50°C

pH	$k_I \cdot 10^{-3}$ (min^{-1})	$k_{II} \cdot 10^{-3}$ (min^{-1})
3.0	0.94	
4.0	0.90	
4.5	1.52	
5.0	1.77	
5.5	3.15	
6.0	5.68	
7.0	10.7	
8.0	11.3	2.20
9.0	11.5	2.74
10.0	11.7	2.53

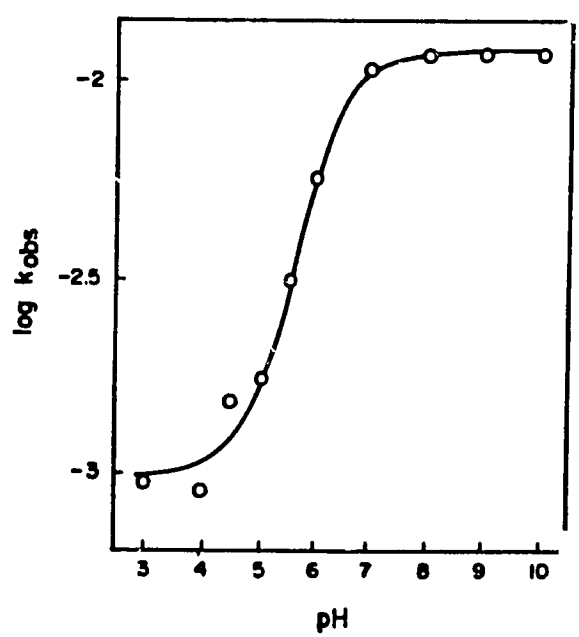


Fig. 2. pH-rate profile for the decomposition of NDIBOA in aqueous solutions at 50°C.

Scheme 2

$$k_{obs} = \frac{k_1 (H^+) + k_2 K}{(H^+) + K}$$

Two likely possibilities for the structure of the intermediate that accumulates between pH 7 and 10 are an isocyanate and a carbamic acid. The following evidence indicates that this latter possibility prevails: i) the UV spectrum of the intermediate is similar to that expected for a carbamic acid[17]; ii) addition of amines to the reaction medium failed to produce any measurable amount of substituted urea; iii) the spectrophotometric changes and the rates observed for the second phase of the reaction were comparable to those reported for the conversion of N-(p-nitrophenyl) carbamate into p-nitroaniline[17]; iv) control runs subjecting 6-nitrobenzoxazolin-2-one to similar reaction conditions failed to produce the corresponding aminophenol. In the reaction of 2,4-dihydroxy-1,4-benzoxazin-3-ones with substituents in position 7 less electron-accepting than the nitro group, internal nucleophilic attack by the phenolic oxygen in the isocyanate prevents the formation of a carbamic acid and leads directly to a 1,3-benzoxazin-2-one. When the 7 nitro group is present, its electron-attracting properties decrease the nucleophilicity of the phenolic oxygen. In this case, nucleophilic attack by solvent brings the rapid formation and accumulation of the corresponding carbamic acid, as has been reported earlier[17,18] (Scheme 1, path B).

At pH lower than ca. 7, the spectral changes do not evidence the accumulation of an intermediate. In this case formation of the intermediate is slower than its further reaction with solvent since this latter reaction is acid catalyzed[19]. However, since the product of the reaction is the same at every pH studied, it is safe to assume that the reaction follows the mechanism described throughout the pH range considered.

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REACTION OF 7-SUBSTITUTED-4-HYDROXYL-1,4-BENZOXAZIN-3-ONES IN STRONGLY ACIDIC MEDIA

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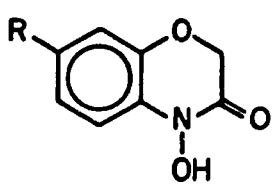
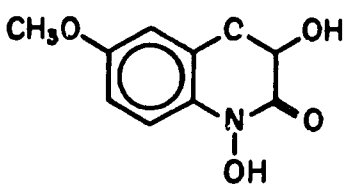
The *in vivo* mutagenicity of certain arylamines has been associated with their enzymatic oxidation to arylhydroxamic acids, further activation forming sulfate esters, and finally to formation of the ultimate carcinogen, a nitrenium ion.[1-3] The 1,4-benzoxazin-3-one derivatives 1a and 2 are mutagenic.[4] The O-acetylated derivative of 1a has been shown to react with nucleophiles,[5] derivatives of nucleic acids among others,[6,7] in reactions postulated to occur with the intermediacy of a nitrenium ion.[8]





Compound	1a	1b	1c	1d	1e	1f	1g	2
R	CH_3O	CH_3	H	F	Cl	COOMe	CN	

The reaction of 1c in HCl to give 7-chloro-1,4-benzoxazin-3-one has been suggested to occur with formation of a nitrenium ion, in a sequence such as 1-3. We report herein kinetic studies on the decomposition of a series of 7-substituted-1,4-benzoxazin-3-ones (1a-1g) in HCl of different concentrations which support the scheme proposed.

$$\text{Ar-NOH-COR} + H^+ \longrightarrow \text{Ar-NOH}_2^+\text{-COR} \qquad (1)$$

$$\text{Ar-NOH}_2^+\text{-COR} \longrightarrow \text{Ar-N}^+\text{-COR} + H_2O \qquad (2)$$

$$\text{Ar-N}^+\text{-COR} \longrightarrow \text{Product} \qquad (3)$$

The required hydroxamic acids 1a-1g were synthesized by reductive cyclization of the corresponding nitroesters, which were obtained by reaction of potassium o-nitrophenoxides with α-bromoacetate. All compounds gave spectral data consistent with the assigned structures.

The main product isolated from the reaction of 1c with concentrated HCl were 5-chloro- and 7-chloro-1,4-benzoxazin-3-one, while for the analogous reaction of 1a it was 5-chloro-7-methoxy-1,4-benzoxazin-3-one.

The kinetics of the reactions were followed spectrophotometrically. At high HCl concentrations, the spectra of the reactants decreased continuously, while at low concentrations, an isosbestic point appeared. In all cases, however, the kinetics of the actions were pseudo-first order with respect to reactants.

Figure 1 shows the rate constants as a function of H_o/pH for the unsubstituted mpound 1c, and suggests independent decomposition of protonated and neutral forms of : compound, in equilibrium with each other as depicted in Scheme 1.

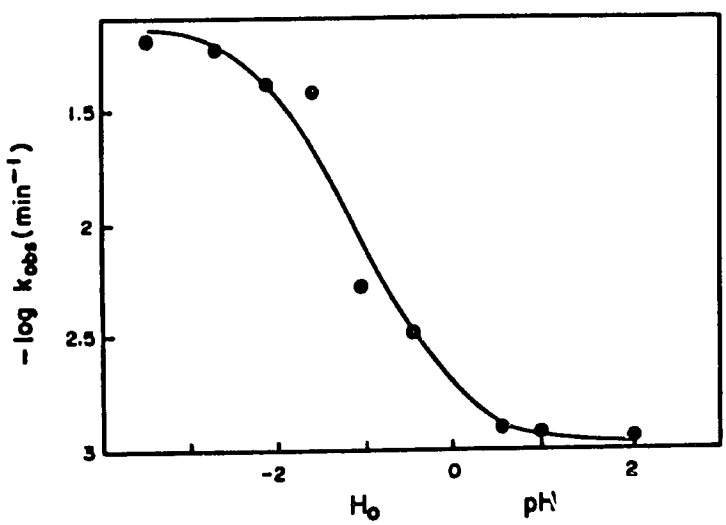
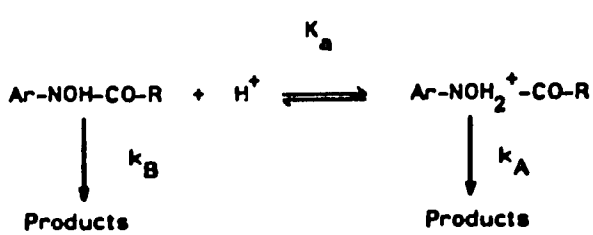


Fig. 1. Decomposition of 1c in HCl of different concentrations at 31°C.

Scheme 1



$$k_{obs} = \frac{k_B + k_A K_a (H^+)}{K_a (H^+) + 1} \qquad (4)$$

105

Experimental points gave a strong fit to equation 4 describing the equilibrium in Scheme 1, and lead to values for k_B, k_A and K_a. pK_a values obtained from similar studies conducted for other compounds of the series generated a linear Hammett plot with values of $\rho(\sigma^+) = 1.42$ and $r = 0.99$. Further evidence for the existence of a protonation equilibrium was obtained from the decomposition reaction of compound 2, a diprotic hydroxamic acid.[9] Reactions followed pseudo-first order kinetics throughout the H_o/pH range studied. The rate constants determined gave rise to a plot against H_o/pH with 3 inflection points (Figure 2), corresponding to the dissociation processes involving protonated, neutral, monoanionic and dianionic species.

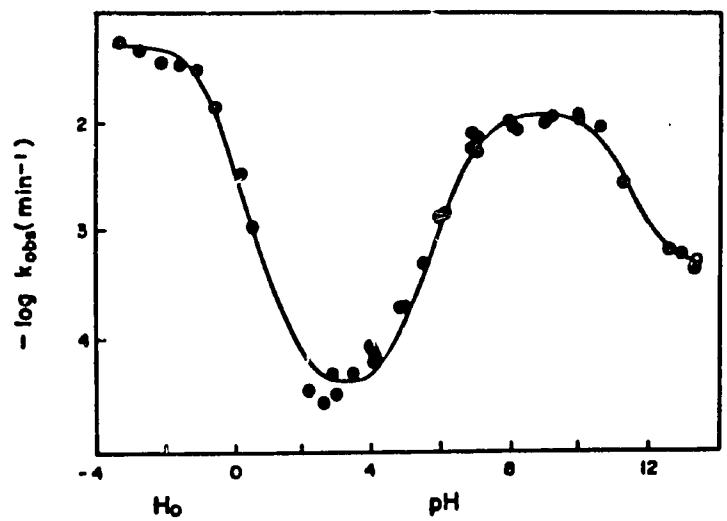


Fig. 2. Decomposition of 2 in HCl of different concentrations (H_o range) or different aqueous buffers (pH range) at 31°C. The line drawn corresponds to a least-squares fit of the experimental points to an extension of equation 4 which takes into account the four possible reactive species: protonated, neutral, monoanionic and dianionic.

The rates of decomposition of protonated 1a-1g (k_A values from equation 4) are plotted against Hammett parameters in Figure 3. The compounds fell into two distinct groups. Those with electron donating substituents were best correlated by Hammett σ parameters, the value of ρ being 0.71, and the correlation coefficient 0.99, as contrasted with 0.86 if σ^+ was used. Compounds with electron withdrawing substituents were best correlated by Hammett σ^+ parameters, the value of ρ being -4.08, and the correlation coefficient -0.96, as contrasted with -0.88 if σ was used.

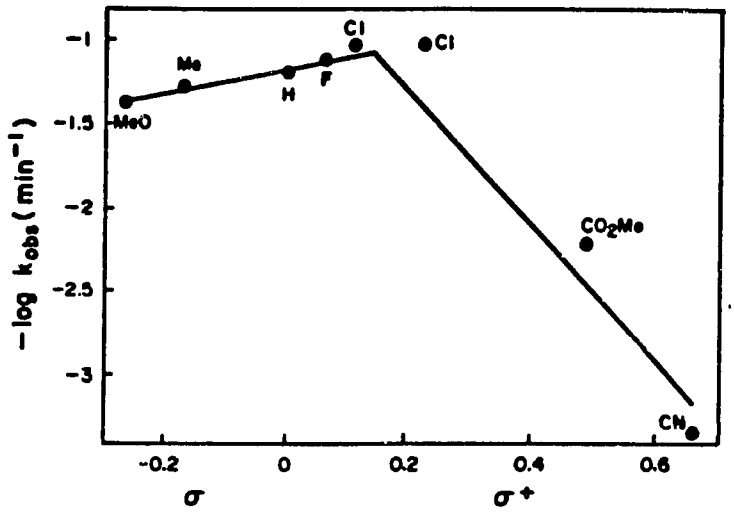


Figure 3. Hammett plot for the decomposition of protonated hydroxamic acids 1a to 1g, at 31°C.

It has been pointed out that Hammett plots with a concave downward break are indicative of a change in the rate determining step of an otherwise constant mechanism.[10] This is consistent with equations 1-3, in which reaction 3 refers to the attack of chloride ion on the aromatic ring. Thus, in compounds with electron withdrawing substituents, the ionization step is rate determining; however, when the substituents stabilize the intermediate nitrenium ion sufficiently through electron release, nucleophilic addition by chloride ion becomes rate determing.

The signs and magnitudes of the ρ values and the nature of the parameters best describing the correlations are in agreement with this interpretation. When step 2 is rate determining, the transition state is electron defficient with respect to the ground state, and ρ is expected to be negative and best correlations are expected with σ^+. The ρ value obtained might be compared with that for the decomposition of methanosulfonate esters of N-hydroxyacetanilides in chloroform (ρ = -9.24 with σ^+) which was shown to proceed through a nitrenium ion.[11] The difference may be accounted for by a better leaving group and a less polar solvent in the case of sulfonate esters. When step 3 is rate determining, nucleophilic attack by chloride ion is facilitated by electron removal from the reaction center and a small positive ρ value is expected.

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Sources of hydroxamic acids, secondary metabolites in wheat confering resistance against aphids

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NIEMEYER, H.M., COPAJA, S.V. and BARRIA, B.N. Sources of hydroxamic acids, secondary metabolites in wheat confering resistance against aphids.

Hydroxamic acids of the 2,4-dihydroxy-1,4-benzoxazin-3-one type (DIBOA; DIMBOA= 7-methoxy derivative) play an important role in the resistance of wheat to aphids, through antibiosis and feeding deterrency. The screening of species of the tribe Triticeae has shown that useful sources of hydroxamic acids exist for breeding programs aimed at increased aphid resistance in wheat. Is is shown that hydroxamic acids may be used as taxonomic markers. On this basis, it is suggested that *Triticum speltoides* is a likely donor of the B genome to hexaploid wheat.

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101

Key words: hydroxamic acids, DIMBOA, DIBOA, Triticeae, wheat resistance to pests, wheat breeding, chemotaxonomy, B-genome origin

110

Hydroxamic acids (Hx) were discovered over three decades ago in relation to fungal diseases of rye (VIRTANEN and HIETALA 1960). Later found in maize, they were associated with resistance to the European corn borer, Ostrinia nubilalis. Breeding programs led to the production of maize cultivars with high Hx levels which were resistant to leaf feeding by the first brood of the borer (GROMBACHER et al. 1986; GUTHRIE et al. 1986).

The first demonstration of an inverse relationship between aphid performance and Hx concentrations concerned Rhopalosiphum maidis growing on different maize inbreds (LONG et al. 1977). Since then, negative correlations have been described between Hx levels both in cultivated and wild Gramineae and performance of cereal aphids determined either as infestation levels of the plants (BECK et al. 1983), population growth rate (ARGANDOÑA et al. 1980; CORCUERA et al. 1982), intrinsic rate of natural increase (BOHIDAR et al. 1986; THACKRAY et al. 1990a) or mean relative growth rate (THACKRAY et al. 1990b).

Similar negative correlations were obtained with Metopolophium dirhodum feeding on excised barley leaves (originally lacking Hx) in which different levels of DIMBOA had been incorporated (ARGANDOÑA et al. 1980). Survival of cereal aphids decreased with increasing DIBOA or DIMBOA concentrations in artificial diets on which aphids fed (CORCUERA et al. 1982; ARGANDOÑA et al. 1983).

Dual choice tests between wheat cultivars differing in DIMBOA levels showed that Rhopalosiphum padi preferentially settled on seedlings with lower DIMBOA levels (GIVOVICH and

NIEMEYER 1991). Electronic monitoring of aphid feeding behavior (TJALLINGII 1990) showed that fewer aphids reached the phloem within a given time, and they required longer times to contact a phloem vessel in wheat seedlings with higher DIMBOA levels (GIVOVICH and NIEMEYER 1991). This feeding deterrent effect has been shown to decrease transmission of barley yellow dwarf virus to wheat seedlings (GIVOVICH and NIEMEYER 1991).

These arguments have led to the proposal of increasing hydroxamic acid levels in wheat plants in order to obtain increased resistance to aphids (NIEMEYER 1988a; WRATTEN et al. 1991). In this paper, results are presented of the screening of a wide range of Triticeae which show the existance of germplasm useful for breeding programs aimed at increasing Hx levels in wheat. The data presented also show the potential of hydroxamic acids as chemotaxonomic markers within the Triticeae, and allow the suggestion of Triticum speltoides as the donor of the B genome of hexaploid wheat.

Materials and methods

Plant material

Seeds have been obtained from Instituto de Investigaciones Agropecuarias (INIA), Chile; Plant Breeding International, Cambridge, UK; United States Department of Agriculture (Small Grains Collection and ARS Crop Research Laboratory) and University of Missouri-Columbia.

Tables 1 through 4 list the Triticeae which have been analysed. Since Hx levels vary with the age of the plant (ARGANDOÑA et al. 1980; ZUÑIGA et al. 1983; THACKRAY et al. 1990a), analyses were carried out on the aerial parts of seedlings at the age of maximal Hx level. This corresponded to 4- to 7-day old seedlings, depending on the accession studied.

The classification and nomenclature of the Triticeae have been the subject of much debate (DEWEY 1984; BAUM et al. 1987; GUPTA and BAUM 1989; JAUHAR and CRANE 1989; KELLOG 1989). In this paper, the names of cultivated Triticum species follow the system of BRIGGLE (1980), of wild Triticum species that of KIMBER (1983), and of perennial Triticeae that of DEWEY (1984).

Analysis of hydroxamic acids

Two different methods were used for Hx analysis: i) a colorimetric method based on the formation of a complex between hydroxamic acids and $FeCl_3$ and its quantification by UV-Vis spectroscopy (BOHIDAR et al. 1986), was used in cultivated Triticum species of Table 2 (NIEMEYER 1988b); ii) a method based on the separation of hydroxamic acids by high performance liquid chromatography and quantification with a UV-Vis detector (NIEMEYER et al. 1989) was employed in the sets of species in Tables 1, 3 and 4 (COPAJA et al. 1991).

Results and discussion

Hydroxamic acids as chemotaxonomic markers

The variations observed both in the total and in the relative concentrations of hydroxamic acids within different taxa in the Triticeae suggest they might be useful taxonomic markers. Support for this hypothesis was provided by data from cereals and from wild *Triticum* species.

While bread wheat (genome ABD) contains higher concentrations of DIMBOA than of DIBOA (COPAJA et al. 1991), and rye (genome R) contains only DIBOA, the man-made hybrid triticale (genome ABDR) contains both DIBOA and DIMBOA (Table 1), thus reflecting its constitutive genomes. Similar effects appear in the comparison of concentrations of DIBOA and DIMBOA in diploid and polyploid wild *Triticum* species. Thus, the C genome of *T. dichasians* (low DIBOA levels), together with the D genome of *T. tauschii* (low DIBOA level) in *T. cylindricum* leads to undetectable DIBOA levels, and together with the U genome of *T. umbellulatum* (Hx not detected) in *T. triunciale* leads to undetectable levels of Hx (Table 3). The capacity of the Un genome of *T. unisristatum* to lead to the accumulation of high levels of DIBOA is also seen in the DUn genome of *T. ventricosum*.

Hydroxamic acids also reflect affinities among the perennial Gramineae (Table 4). Thus, the genera *Elymus* and *Leymus*, which constitute a single genus in the traditional treatment of the tribe (HITCHCOCK 1951), show high DIBOA levels. *Agropyrum*,

Pseudoroegnaria and Elytrigia, frequently considered as part of the genus Agropyrum (DEWEY 1984; KELLOG 1989), show low levels of both hydroxamic acids. A similar situation occurs in Thinopyrum, considered in some treatments together with Pseudoroegnaria and Elytrigia (TZVELEV 1976).

Hexaploid wheat is an amphiploid containing genomes A, B and D (KERBY and KUSPIRA 1987). While it is clear that T. tauschii is the donor of the D genome (GILL and KIMBER 1974; FERNANDEZ DE CALEYA et al 1976; JONES et al. 1976) and T. monococcum that of the A genome (FERNANDEZ DE CALEYA et al. 1976; JONES et al. 1982), there has been much debate over the origin of the B genome. There is agreement that the putative donor belongs to the Sitopsis section of the genus (S genome). However, different techniques have led to the proposal in turn of each of the species of the section as the most likely candidate: T. speltoides (SARKAR and STEBBINS 1956; RILEY et al. 1958; REES and WALTERS 1965; JAASTS 1978; BAHRMAN et al. 1988), T. bicorne (SEARS 1956), T. sharonense (KUSHNIR and HALLORAN 1981), T. longissimum (VITTOZI and SILANO 1976; GERLACH et al. 1978; TSUNEWAKI and OGIHARA 1983) and T. searsii (FELDMAN 1978; NATH et al. 1983, 1984; THOMPSON and NATH 1986). Tetraploid wheats (genome AB) accumulate high levels of DIMBOA and low levels of DIBOA (COPAJA et al. 1991b). Since the A genome of T. monococcum leads to the accumulation of low levels of both hydroxamic acids (Table 2), it seems unlikely that the donor of the B genome is T. bicorne, T. longissimum or T. searsii, which produce mainly DIBOA, in relatively low quantities (Table 3). T. sharonense

115

contains only low concentrations of DIMBOA and hence seems also an unlikely candidate. The data presented in Table 3 point to T. speltoides as the most likely putative donor of the B genome of wheat, since it contains high levels of DIMBOA. The nature and quantity of hydroxamic acids found in species of the Sitopsis section allow a separation into T. speltoides (high DIMBOA levels) on one hand, and T. bicorne, T. sharonense, T. longissimum and T. searsii (low DIBOA and DIMBOA levels) on the other. This finding agrees with earlier dendrograms based on similarities of two-dimensional gel electrophoresis of seedling proteins of species of the Sitopsis section (BAHRMAN et al., 1988). The lower levels of hydroxamic acids in hexaploid wheat relative to durum wheats might be attributed to the low levels found in T. tauschii, the donor of the D genome of hexaploid wheat.

Sources of hydroxamic acids for wheat breeding

Within the common wheats, durum wheats showed substantially higher concentrations of Hx than did bread wheats (Table 2). Given the similar toxicity of both hydroxamic acids for aphids (ZUÑIGA et al. 1983), the genome of rye seems a promising candidate for the transfer of aphid resistance to wheat.

Particularly interesting genomes as sources of hydroxamic acids are the S genome of T. speltoides (DIMBOA) and the Un genome of T. unisristatum (DIBOA). Crosses have been shown to be

possible between wheat and all the perennial genera of the Triticeae, with the exception of Psathyrostachys. This has allowed the transfer of useful agronomical traits to wheat (SHARMA and GILL 1983; DVORAK et al. 1985; STOREY et al. 1985). This genus seems particularly promising as a source of DIBOA. Further work should be directed to the possibility of crossing this species with wheat.

The use of Hx as aphid resistance factors presents as potential limitation the variation of their whole-plant concentration as the plant develops: after a steep increase soon after germination, it decreases considerably (ARGANDOÑA et al. 1981; THACKRAY et al. 1990a). However, it has been shown repeatedly, that the youngest tissue in a wheat plant retains a high concentration of Hx (ARGANDOÑA et al. 1980; ARGANDOÑA et al. 1981; ZUÑIGA et al. 1983; NIEMEYER 1988b; THACKRAY et al. 1990a; COPAJA et al. 1991a). The concentration of Hx in areas of sensitive new growth may have important consequences for defence against aphids such as Sitobion avenae which feeds on flag leaves and ears (WRATTEN 1975) and R. padi which is a seedling pest in western Europe (VICKERMAN and WRATTEN 1979). A negative correlation has been found between mean relative growth rate of S. avenae and Hx concentration in flag leaves of wheat plants (LESZCZYNSKI et al. 1989).

Emergence of resistant biotypes of aphids is a common feature of aphid-host plant interactions. Hence, a breeding program of wheat aimed at aphid resistance should be based on a broad range of factors which confer stability to the overall

117

resistance. Non-glaucousness has been described as an antixenotic factor in wheat against S. avenae (LOWE et al. 1985). Phenols have been shown to play a role in wheat resistance against S. avenae (LESZCZYNSKI et al. 1989). Incorporation of these characteristics, together with high levels of hydroxamic acids, should be among the aims of wheat breeding programs (WRATTEN et al. 1991).

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121

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125

Table 1. Hydroxamic acid levels in cereals

Cereal	n*	Mean concentration DIBOA (mmol/kg fr. wt)	Mean concentration DIMBOA (mmol/kg fr. wt)
Wheat	52	0.13 ± 0.19	4.0 ± 2.1
Rye	4	15.4 ± 13.9	ND
Triticale	11	2.1 ± 1.4	2.4 ± 1.5
Barley	6	ND	ND

*n = number of cultivars analyzed. Mean ± standard error.
ND = not detected.

Table 2. Hydroxamic acid levels in cultivated *Triticum* species#

Species	Genome	n*	Mean Hx concentration (mmol/kg fr. wt)
T. monococcum§	A	4	0.6 ± 0.58
T. araraticum	AG	2	2.8 ± 0.71
T. timopheevi	AG	4	2.6 ± 0.74
T. zhukovskyi	AG	2	1.8 ± 0.13
T. carthlicum	AB	3	3.8 ± 0.43
T. dicoccon	AB	4	5.1 ± 0.96
T. polonicum	AB	4	5.4 ± 0.94
T. turgidum	AB	5	5.7 ± 2.46
T. compactum	ABD	5	1.9 ± 0.55
T. macha	ABD	3	1.5 ± 0.91
T. spelta	ABD	5	2.5 ± 0.58
T. sphaerococcum	ABD	5	3.1 ± 0.44

* number of accessions analyzed. Mean ± standard error.

data mainly from Niemeyer, 1988a.

§ HPLC analysis of 2 accessions showed concentrations of DIBOA and DIMBOA of 0.14 and 0.08 mmol/kg fr. wt, respectively.

Table 3. Hydroxamic acid levels in wild *Triticum* species#

Species	Genome	n*	Mean concentration DIBOA (mmol/kg fr. wt)	Mean concentration DIMBOA (mmol/kg fr. wt)
T. tripsacoides	Mt	1	ND	ND
T. speltoides	S	3	ND	5.59 ± 1.55
T. bicorne	S^b	4	0.25 ± 0.32	TR
T. sharonense	S	2	ND	0.17 ± 0.04
T. longissimum	S^l	4	0.48 ± 0.44	TR
T. searsii	S^s	2	1.27 ± 1.00	TR
T. dichasians	C	2	0.15 ± 0.15	ND
T. comosum	M	1	ND	1.09
T. uniaristatum	Un	1	3.78 ± 0.92	ND
T. umbellulatum	U	3	ND	TR
T. ovatum	UM	2	ND	0.40 ± 0.03
T. macrochaetum	UM	2	TR	TR
T. columnare	UM	2	ND	ND
T. triunciale	UC	2	TR	TR
T. kotschyi	US	2	2.25 ± 1.11	0.30 ± 0.16
T. tauschii	D	3	0.18 ± 0.15	TR
T. cylindricum	DC	2	ND	ND
T. ventricosum	DUn	2	6.00 ± 0.42	ND
T. crassum	DDM	2	ND	ND
T. juvenale	DMU	2	ND	ND
T. syriacum	DMS	2	ND	ND

limit of detection of the method: 0.1 mmol/kg fr wt.; TR = traces; ND = not detected.

* number of accessions analyzed. Mean ± standard error.

-128-

Table 4. Hydroxamic acid levels in perennial Triticeae*

Genus	n§	m#	Mean concentration DIBOA (mmol/kg fr. wt)	Mean concentration DIMBOA (mmol/kg fr. wt)
Agropyrum	4	15	0.24 ± 0.18	0.22 ± 0.07
Critesion	7	19	2.27 ± 1.97	0.37 ± 0.18
Elymus	9	10	5.61 ± 4.51	0.40 ± 0.46
Elytrigia	1	2	0.11	0.26
Hordeum	5	5	1.25 ± 0.77	ND
Leymus	7	11	3.27 ± 3.02	1.08 ± 0.74
Secale	1**	3	31.14 ± 10.45	ND
Thinopyrum	6	13	0.68 ± 0.52	0.86 ± 0.53
Pascopyrum	1	3	0.14	1.13
Psathyrostachys	2	4	14.45 ± 5.98	ND
Pseudoroegnaria	3	10	0.97 ± 0.26	0.12 ± 0.12

* data mainly from Copaja *et al.*, 1991a.

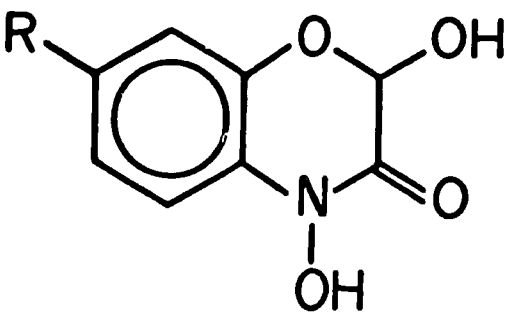
§ number of species analyzed. Mean ± standard error.

\# number of accessions analyzed.

** includes 3 subspecies of *S. montanum*.

ND = not detected.

129



DIBOA : R = H

DIMBOA : R = CH_3O

EFFECT OF DIMBOA, AN APHID RESISTANCE FACTOR IN WHEAT, ON THE APHID PREDATOR ERIOPIS CONNEXA GERMAR (Col.: Coccinellidae)

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Abstract-DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one), a secondary metabolite found in cereal extracts, confers resistance in wheat to aphids. Its effect on beneficial organisms was tested on larvae of the aphid predator Eriopis connexa Germar. Larvae were fed until pupation on artificial diets to which different concentrations of DIMBOA (2 to 200 μg/g diet) were added, as well as on aphids which had been feeding on wheat seedlings with different DIMBOA levels (140 to 440 μg/g fresh tissue). In diets, the effect of DIMBOA was greatest on survival of third instar larvae and on the duration of the second and fourth instars. When aphids were provided as food, those which had fed on a wheat cultivar with intermediate DIMBOA level led to a significantly longer larval duration in the predator than did those which fed on either low or high DIMBOA cultivars. Shortest predator development times were obtained with aphid prey that had fed on high DIMBOA seedlings. Higher DIMBOA levels in the plant appear to reduce aphid feeding rates (and rates of DIMBOA ingestion) decreasing aphid survival and minimizing the effect of the toxin on the predator.

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131

Key words: wheat, hydroxamic acids, DIMBOA, plant resistance, cereal aphids, aphid predators, Eriopis connexa, tri-trophic interactions,

INTRODUCTION

The most prevalent cereal aphids in Chile are Metopolophium dirhodum (Walk.), Sitobion avenae (Fab.), Schizaphis graminum (Rond.) and Rhopalosiphum padi (L.) (Ramírez, 1990). Although an array of natural enemies which include predators, parasitoids and pathogens limit, to a great extent, their populations (Zúñiga, 1987), aphids constitute a potential problem due to their ability to transmit virus diseases, such as the barley yellow dwarf virus (BYDV) (Herrera and Quiroz, 1988). Eriopis connexa Germar (Coccinellidae) is one of the most important aphid predators in Chile because of its high populations in spring and summer, wide range of prey, and predatory efficiency (Macfarlane, 1985).

DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one), the main hydroxamic acid (Hx) present in wheat extracts (Niemeyer, 1988a), has been associated with resistance of the plant to aphids through antibiosis (Argandoña et al., 1980; Bohidar et al., 1986; Thackray et al., 1990; Wratten et al., 1991) and feeding deterrency (Argandoña et al., 1983; Niemeyer et al., 1989b; Givovich and Niemeyer, 1991).

Biological control and host plant resistance are often compatible and even complementary (van Emden and Wratten, 1991), as has been found to be the case of resistance in varieties of barley and sorghum which complemented the activity of the

132

parasitoid Lysiphlebus testaceipes Cresson (Starks et al., 1972). However, variations in host plants may have dramatic effects on biological control agents. Thus, prey confined to resistant host plants may represent a low-quality food for predators and parasitoids, affecting their biology (Bergman and Tingey, 1979). Secondary metabolites in the plants may be the cause of detrimental effects on organisms at the third trophic level, as is the case of α-tomatine, an alkaloid in Solanum and Lycopersicon species, which is toxic to the endoparasitoid Hyposoter exiguae (Viereck) which takes up the compound through its host Heliothis zea (Boddie) (Campbell and Duffey, 1979). Nicotine, an alkaloid from Nicotiana species confers plant resistance to Manduca sexta L. but has negative effects on the parasitoids Hyposoter annulipes (Cresson) and Cotesia congregata (Say) developing on M. sexta larvae reared in nicotine-containing diets (Thorpe and Barbosa, 1986; Barbosa et al., 1986). Plant secondary metabolites might confer resistance in the herbivore towards the predator, as is the case of Macrosiphon albifrons, an aphid which is protected from attack by Carabus problematicus by quinolizidine alkaloids ingested from its lupin host plants (Wink and Römer, 1986).

An increase in Hx levels in wheat, proposed as a mechanism to enhance resistance towards aphids (Niemeyer, 1988b; Copaja et al., 1991a, 1991b; Wratten et al., 1991), could affect beneficial organisms. Hence, the effect of DIMBOA on the development of E. connexa was examined.

MATERIALS AND METHODS

A mass rearing technique and an artificial diet for *Eriopis connexa* were developed; the direct effect of DIMBOA was studied by adding the compound in different concentrations to the diet; finally, larvae were fed aphids reared on wheat lines containing different DIMBOA levels.

Mass rearing of E. connexa. Rearing was started with 25 pairs of coccinellids collected in fields near the laboratory. Beetles were fed on the aphid *R. padi* reared on oat seedlings in a room maintained at 21°C with a 10°C range, 50% R.H. with a 10% range and L16:D8 photoregime.

All stages of the predator were maintained separately in plastic containers of 12 cm diameter and 7.5 cm height fitted with a cover with fine holes. Adults were confined at a density of 5 to 8 pairs per container. Aphids on oat leaves were placed as food on a sheet of paper towel, inside the container. Egg groups were collected daily and maintained separately in containers. Larvae were also fed with aphids from oats; these aphids were offered as soon as egg hatch occurred. The fourth instars were supplied with a sheet of paper towel for pupation. The mean number of aphids provided daily per larva from first to fourth instar were 10, 25, 20 and 15, respectively. Pupae were collected daily and were placed on a sheet of paper towel inside a container. Adults which had emerged within the same 24-hour period were sexed and maintained in pairs in the containers.

Artificial diet for E. connexa. Diets were based on those developed for coccinellids using liver and meat extracts (Herrera, 1960; Atallah and Newson, 1966; Ferrand and Laforge, 1975) and were tested by rearing first instar larvae in 5.5 cm diameter Petri dishes, each larva receiving 0.5 g diet every two or four days. Table 1 shows the best formulation of those tested.

Pollen with polyvitamins and wheat germ were pulverized separately with mortar and pestle. These constituents were mixed with sorbic acid, ascorbic acid and vitamin E and then bee honey was added. This mixture was blended for 30 sec. with liver in 50 ml water in a domestic blender. Agar was dissolved in 50 ml water at 80°C, and cooled down before being mixed into the previous mixture for one minute. The homogeneous dough of semi-solid texture obtained was dispensed to sterilized containers and stored at 6°C.

The diet was tested with 10 first-instar larvae and was renewed every two days. For comparison, 14 first-instar larvae were reared individually with natural diet using R. padi nymphs ad libitum as food.

Effect of DIMBOA in artificial diets on the development of E. connexa. First instar larvae of E. connexa were reared on artificial diet containing DIMBOA, isolated from ethereal extracts of Zea mays cv. T129s, as previously described (Queirolo et al., 1983). This compound was incorporated to the diet at the required concentration in 50 ml of water before adding the agar suspension. The latter was cooled down before use in order to

minimize decomposition of DIMBOA. Analysis of the diet after four days at room temperature reveiled that at least 85% of DIMBOA remained in it. Experiments were carried out in a completely randomized design with six treatments and two replicates. Treatments consisted of five DIMBOA concentrations in artificial diet plus a control without DIMBOA. Concentrations tested were 0, 2, 5, 10, 50 and 200 μg DIMBOA/g diet. Each replicate consisted of ten recently-hatched larvae of E. connexa maintained separately in 5.5 cm diameter Petri dishes. The diet (0.5 g) was renewed every four days for the first and second instars and every two days for the third and fourth instars. The Petri dishes were sealed with Parafilm to keep larvae from escaping and diet from drying. Larval mortality, duration of each instar, pupal mortality, pupal duration, weight of pupae and time to reach adulthood were evaluated.

Study on E. connexa larvae fed on aphids reared on DIMBOA-containing wheat lines. Three wheat lines with different contents of the compound were chosen (Millaleu: 140 μg DIMBOA/g fresh tissue; Nobo: 270 μg and Maiten: 440 μg). Oats were used as a control without DIMBOA. Aphids (R. padi) were allowed to feed on the plants for 24 hours. They were placed within a clip cage located in the middle third of the leaf of a wheat seedling in the 1-leaf stage, the stage at which DIMBOA was analyzed. One first-instar larva of E. connexa was placed inside each Petri dish with a piece of wet filter paper and allowed to develop to the adult stage. Aphids were removed from the plants and placed

inside the Petri dish ad libitum for the larvae. For each wheat cultivar and the oat control there were ten replicates. Survival of larvae, number of aphids eaten per larva, and the duration of each larval developmental stage were determined. The larvae employed were obtained from eggs laid by field-collected females which had been kept together with males for two weeks under the rearing conditions described above.

Plant material and hydroxamic acid analysis. Wheat seedlings used were in the 1-leaf stage (G.S. 11; Zadoks et al., 1974). Hydroxamic acids were analyzed by reverse phase high performance liquid chromatography, as previously described (Niemeyer et al., 1989a).

RESULTS

Mass rearing of E. connexa. This technique was developed in order to obtain uniform individuals in high quantities for laboratory assays. Mating was frequent and occurred from early adulthood up to just before death. Females began oviposition on average 16.5 days after reaching adulthood and the oviposition frequency was every three to four days. Eggs were laid in groups, usually on the surface of paper towel, each batch comprising c. 25 eggs. Individuals older than three months laid a higher proportion of infertile than fertile ones. Eggs were collected daily by cutting the paper around the batch; they hatched five days later.

Larvae were voracious, consuming on average 54, 39, 55 and 72 nymphs of R. padi in first, second, third and fourth instars, respectively. Development times for each stage are given in Table 2. The pupal stage lasted 5.4 days and the sex ratio was 1:1. These adults consumed 15 to 20 aphids per day. The life cycle was completed in 44 days.

Artificial diet for E. connexa. The rearing of E. connexa under laboratory conditions is relatively easy and cheap with aphids as food. However, experiments in which the effect of a given compound is being investigated demand the use of an artificial diet for rearing. Table 2 shows comparative data on larval and pupal development of E. connexa on natural and artificial diets. Larvae were reared on artificial diets from first instar to adulthood. Moulting was normal with no mortality recorded. However, the larval developmental period was different from that observed with natural diet and adults, although being as large as the controls, were whitish in color, possibly due to a lack of appropriate pigments in the diet.

Effect of DIMBOA in an artificial diet on the development of E. connexa. Table 3 shows the effect of DIMBOA in an artificial diet on the development and survival of larvae of E. connexa. Survival was not significantly affected by DIMBOA, with the exception of that of the third instar larva. In this case, progressively lower survival was obtained with diets with higher DIMBOA concentrations.

The duration of instars two and four were significantly increased by higher DIMBOA concentrations in the diet, albeit only at the highest concentration in the latter instar.

Table 4 shows the overall effect of DIMBOA on larval development and survival, and also the effect on pupae and adults obtained from treated larvae. While an increase in time to reach adulthood seems apparent at the lowest concentration of DIMBOA tested, the effect was statistically significant only for the highest concentration of DIMBOA. This concentration also decreased larval survival but had no effect on pupal survival nor on adult emergence.

Effect of aphids reared on DIMBOA-containing wheat lines on E. connexa larvae. The development time of E. connexa larvae differed significantly depending on the wheat cultivar on which their aphid prey had fed, the duration being longer in the case of the cultivar with an intermediate level of DIMBOA. Pupal developmental time was not affected by the level of DIMBOA in the wheat cultivar. Larval survival was lower with aphids that had fed on the wheat cultivar with intermediate DIMBOA level (Table 5); aphid consumption was also lower in this cultivar (Table 6).

DISCUSSION

DIMBOA has been shown to be a feeding deterrent towards aphids. Thus, survival of aphids feeding on diets (Argandoña et al., 1983; Niemeyer et al., 1989b) or on wheat plants (Niemeyer

et al., 1989b) differing in DIMBOA level showed a biphasic behavior: in the lower DIMBOA range survival was inversely correlated with DIMBOA concentrations while at the higher end survival was positively correlated with DIMBOA concentrations. Dual choice tests showed that aphids preferentially settled on leaves of wheat seedlings containing lower DIMBOA levels (Givovich and Niemeyer, 1991). Finally, electronic monitoring of aphid feeding behavior showed that fewer aphids reached the phloem and those that did took longer to reach it in seedlings with higher DIMBOA levels than in those with lower ones (Givovich and Niemeyer, 1991). Hence, it appears that below a given DIMBOA level, the aphid ingests the compound, while above this level DIMBOA deters the aphid from feeding, leading to higher short-term survival rates.

An aphid feeding for 24 h on a wheat seedling will accumulate DIMBOA in concentrations of the order of 1 μg/g aphid (Niemeyer et al., 1989b). Longer feeding times, as may occur under field conditions, should not radically affect this estimation since: i) DIMBOA levels in the plant decrease as the plant develops (Argandoña et al., 1981), thus exposing the aphid to lower DIMBOA levels when feeding; ii) the instability of DIMBOA in aqueous solutions (Niemeyer et al., 1982) will hinder its acumulation; and iii) metabolization of DIMBOA by the insect may occur, as has been shown in the case of a lepidopteran (Campos et al., 1989), which would also hinder its accumulation.

The lowest concentration of DIMBOA in diets tested on E. connexa is comparable with that expected in aphid bodies

(Niemeyer et al., 1989b). This concentration led to a detectable albeit non-significant increase of larval and pupal time period of E. connexa with respect to control diet (Tables 3 and 4). Whe the predator fed on aphids, development times were also altered being longest when the aphid prey had fed on the cultivar whic had an intermediate DIMBOA level. An explanation of this findin is that these aphids were more deleterious to the predator. Thi is supported by the facts that: i) two other cereal aphi species, M. dirhodum and S. avenae feeding on wheat seedling with intermediate DIMBOA levels accumulated the most DIMBOA i their bodies (Niemeyer et al., 1989b), and ii) R. padi feeding o wheat seedlings with intermediate DIMBOA levels accumulated th most DIMBOA (in glucosylated form) in their honeydew (Givovich e al., 1992). Alternatively, longer development times may hav resulted from larvae ingesting fewer aphids (Table 6), whic would still suggest that the compound in the plant was acting o the third trophic level, in this case through feeding deterrency The fact that the development time of the predator significantl decreased when it fed on aphids from wheat seedlings containin high DIMBOA levels with respect to those containing low DIMBO level suggests that an increase in DIMBOA concentration in th plant, useful for aphid resistance, could result in th additional benefit of minimizing the effect of the toxin on th predator. This is a subtle and hitherto un-reported mechanism i a tri-trophic interaction between plant, herbivore and predato (see van Emden & Wratten, 1991).

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144

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146

Table 1. Composition of artificial diet for *E. connexa*

Component	Quantity (g)
Cooked pig liver	68.1
Pollen	10.0
Honey	10.0
Wheat germ	5.0
Agar	4.0
Polyvitamins	1.0
Sorbic acid	1.0
Ascorbic acid	0.5
Vitamin E	0.4
Water	100.0

Table 2. Comparative development times for larvae and pupae of *Eriopis connexa* in natural and artificial diets

		Time period (days)							Survival (%)	
		Larvae					Pupae	L-Ad	Larvae	Pupae
Diet	n*	I	II	III	IV	Total		Total		
R. padi	14	2.9	2.2	4.3	7.6	17.0	5.4	22.4	100	100
Diet	10	5.4	4.7	5.4	9.0	24.5	5.6	30.1	100	100

* Number of individuals studied

Table 3. Effect of DIMBOA on larvae of *Eriopis connexa* reared on an artificial diet

Treatments	I instar		II instar		III instar		IV instar	
µg DIMBOA/ g diet	Duration (days)	Survival (%)	Duration (days)	Survival (%)	Duration (days)	Survival (%)	Duration (days)	Survival (%)
0	6.2 a	95.0 a	7.3 a	95.0 a	7.0 a	94.5 a	8.7 a	83.0 a
2	5.3 a	90.0 a	8.7 ab	89.0 a	9.3 a	81.3 ab	9.3 a	85.0 a
5	5.0 a	90.0 a	9.0 ab	100.0 a	8.4 a	60.0 b	8.8 a	100.0 a
10	5.2 a	80.0 a	9.1 ab	90.0 a	7.5 a	79.0 ab	8.8 a	100.0 a
50	6.3 a	100.0 a	10.4 bc	100.0 a	7.9 a	73.5 b	8.2 a	94.0 a
200	7.3 a	95.0 a	12.4 c	84.5 a	9.3 a	18.8 c	14.0 b	75.0 a

The experiment was begun with 20 first instar larvae. Means within a column followed by the same letter are not significantly different (P = 0.05; Duncan's multiple range test).

Table 4. Effect of DIMBOA on the life cycle of *Ostrinia nonnexa* in artificial diets

Treatments	Larvae		Pupae			Adult	
µg DIMBOA/ g diet	Duration (days)	Survival (%)	Duration (days)	Survival (%)	Weight (mg)	Time to reach adulthood (days)	Emergence (%)
0	29.2 a	70 a	4.4 a	100	7.0 a	33.6 a	100
2	32.[illegible] a	55 a	4.8 a	100	6.1 a	37.3 a	100
5	31.0 a	55 a	4.5 a	100	6.5 a	35.5 a	100
10	30.6 a	55 a	4.3 a	100	6.1 a	34.8 a	100
50	32.8 a	60 a	5.3 a	100	6.7 a	38.0 a	100
200	43.0 b	10 b	4.5 a	100	6.1 a	47.5 b	100

The experiment was begun with 20 first instar larvae. Means within a column followed by the same letter are not significantly different (P = 0.05; Duncan's multiple range test).

Table 5. Development times of larvae and pupae of *E. connexa* reared with aphids (*R. padi*) fed on oats and wheat lines containing different DIMBOA levels

		Development time (days)							Survival	
		Larvae					Pupae	Lv-Ad	Larvae	Pupae
Plant	DIMBOA (µg/g fr. wt.)	I	II	III	IV	Total		Total		
Oat	---	2.1a	1.8a	2.1a	4.4a	10.4a	3.9a	14.3b	100	100
Millaleu	140	2.0a	2.0a	2.8b	3.6b	10.4a	4.0a	14.4b	98	100
Nobo	270	2.3b	1.8a	2.3a	4.3ab	10.7b	4.0a	14.7a	93	100
Maiten	440	2.5b	1.9a	2.3a	2.4c	9.1c	3.8a	13.9c	100	100

The experiment was began with 10 first instar larvae. Means within a column followed by the same letter are not significantly different (P = 0.05; Duncan's multiple range test).

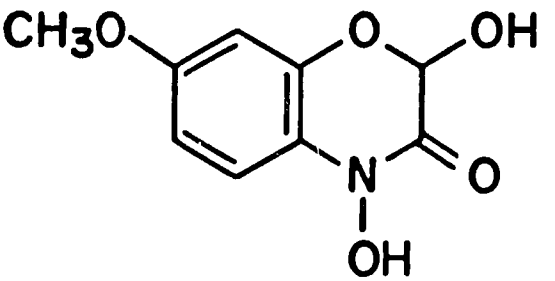
151

Table 6. Aphid consumption by *E. connexa* larvae of aphids reared on oats and on wheat lines containing different levels of DIMBOA.

Plant	DIMBOA (µg/g fr wt)	Instar I	Instar II	Instar III	Instar IV	Total
Oats	-----	14.3ab	39.0a	27.6a	34.3a	115.2a
Millaleu	140	14.5a	38.8a	26.0b	33.7a	113.0b
Nobo	270	14.4ab	37.8b	26.3b	31.6c	110.1c
Maiten	440	14.0b	38.2b	26.2b	32.7c	111.5d

The experiment was begun with 10 first instar larvae. Means within a column followed by the same letter are not significantly different (P = 0.05; Duncan's multiple range test).

154



DIMBOA

HYDROXAMIC ACID GLUCOSIDES IN THE HONEYDEW OF APHIDS FEEDING ON CEREALS, AND THEIR RELATION TO APHID GROWTH RATES

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Running title: Hydroxamic acids in aphid honeydew

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154

Abstract - DIMBOA-glucoside, the main hydroxamic acid (Hx) in intact wheat and maize plants, was detected in the honeydew of Rhopalosiphum padi feeding on seedlings of six wheat cultivars which differed in their concentration of Hx, suggesting that the chemical circulates in the phloem. Neither the aglucone (DIMBOA) nor its breakdown products were found in any of the honeydew samples. Honeydew production by aphids caged on seedlings of the wheat cultivars and DIMBOA-glucoside concentrations in the honeydew followed "bell shaped" curves when plotted against Hx concentration, suggesting passive ingestion of the chemical from the phloem at low Hx concentrations and limited ingestion due to feeding deterrency by Hx in mesophyll cells at high Hx concentrations. The growth rate of R. padi was significantly higher on the high Hx maize variety B49 relative to the low Hx variety B37. This may be attributed to different amino acid composition of the phloem sap of these two varieties, which may affect their nutritional quality and their capacity to alter the toxicity of Hx, or to whole-leaf concentrations of Hx not being directly related to phloem concentrations of Hx. The presence of plant toxins such as Hx glucosides in the phloem sap, the main ingesta of aphids, has major implications for plant defence. The presence of the toxin in the mesophyll may have a deterrent effect during stylet penetration, while its presence in the phloem sap may also provide deterrency (antixenosis) along with antibiosis.

Key words-Rhopalosiphum padi, wheat, maize, DIMBOA, hydroxamic acids, aphid honeydew

15

INTRODUCTION

Hydroxamic acids (Hx) are present in a number of Gramineae (Niemeyer, 1988a, 1988b; Zúñiga et al., 1983; Copaja et al., 1991a; Barría et al., 1991; Copaja et al., 1991b; Niemeyer et al., 1991), such as maize and wheat. Hx exist in the intact plant as glucosides from which the more toxic aglucone is released upon disruption of the plant tissue and contact with a ß-glucosidase (Hofman and Hofmanova, 1969). The most common Hx in maize and wheat tissue is 2-O-ß-D-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA glucoside, Niemeyer, 1988a).

Hydroxamic acids are believed to play a role in the resistance of the plants to pests and diseases (Niemeyer, 1988a). Thus, a number of workers have reported negative correlations between the Hx content of maize and wheat and the performance of insect herbivores feeding on the plants. Most of the work has been concerned with the effects of Hx on the lepidopteran pests of maize, the European corn borer (Klun and Robinson, 1969; Campos et al., 1989) and the western corn rootworm (Xie et al., 1990), and on cereal aphids (Long et al., 1977; Argandoña et al., 1980; Beck et al., 1983; Bohidar et al., 1986; Thackray et al., 1990; Givovich and Niemeyer, 1991).

The concentration of hydroxamic acids is higher in the vascular bundles than in the mesophyll (Argandoña and Corcuera, 1985; Argandoña et al., 1987). However, there is no clear evidence that Hx circulate in the phloem. Thus, although

156

Niemeyer et al. (1989b) found DIMBOA in the bodies of aphids feeding on wheat, Molyneux et al. (1990) failed to find 6-methoxy-benzoxazolin-2-one (MBOA, a breakdown product of DIMBOA) in the honeydew of Schizaphis graminum feeding on wheat.

The aim of the experiments reported here was to determine whether DIMBOA and/or its glucoside could be detected in the honeydew of aphids feeding on wheat, and whether whole-leaf Hx concentration could be related to aphid growth rates in maize.

MATERIALS AND METHODS

Determination of Hx and DIMBOA in plant material. The determination of Hx in maize samples was carried out using a colorimetric method based on the formation of a complex between Hx and $FeCl_3$ (Bohidar et al., 1986; Thackray et al., 1990). This technique produces results which are comparable with those obtained by chromatography (Woodward et al., 1979a and 1979b; Zúñiga et al., 1983).

High performance liquid chromatography (HPLC) was employed for DIMBOA analysis in wheat. The method was essentially that described by Niemeyer et al. (1989a).

Determination of DIMBOA and its glucoside in aphid honeydew. Aphid honeydew was analyzed for DIMBOA and DIMBOA glucoside using HPLC. A standard of DIMBOA glucoside was obtained as described by Lyons et al. (1988).

Insect material. For experiments with maize, aphids were taken from a non-clonal culture of *Rhopalosiphum padi* on barley (cv. Golden Promise), a plant which lacks Hx, in constant temperature Perspex cabinets (Scopes *et al.*, 1975). For experiments with wheat, aphids were reared on oats (cv. Nahuen), a plant also lacking Hx, in a chamber at 22°C with a 6° range.

Presence of DIMBOA-glucoside in the honeydew of R. padi feeding on wheat. The wheat cultivars employed were chosen to contain a wide range of Hx concentrations. Experiments were carried out with seedlings at the one-leaf stage, when Hx levels are highest (Argandoña *et al.*, 1980). Oats (cv. Nahuen) was used as a control without Hx. Twenty adult aphids were placed in a clip cage (2 cm diameter) fitted with a disc of aluminium foil at the bottom and clipped to the abaxial surface of a leaf of a wheat or oat seedling. After 36 h the cage was detached from the plant and the aluminium disc weighed. After washing sucessively with methanol and water and drying, the disc was weighed again. The difference between these measurements was attributed to honeydew produced. The methanol-water washings were concentrated to dryness, redissolved in 200 µl of water and analyzed by HPLC. Three sets of experiments, each consisting of 7 clip cages were performed.

Maize leaf Hx concentration and the growth rate of R. padi. Maize (varieties B37 and B49) was grown in a glasshouse kept at

approximately 20°C and a L16:D8 photoperiod until ready to be used. Aphids (2nd instar nymphs) taken from the main culture were weighed and individually caged in the centre of the second leaf of each of twenty plants in the two-leaf stage (G.S. 12; Zadoks et al., 1974). After aphid placement, the plants were transferred to a growth cabinet (20°C and constant light). Aphid weights and mortality were recorded at 24 h intervals. The weights were used to calculate the mean relative growth rate (M.R.G.R.), a parameter highly correlated in this species with the intrinsic rate of increase (r_m; Leather and Dixon, 1984).

When the last aphid weight had been taken (after 96 h), the leaves upon which the aphids had been caged were sampled and Hx determined by the colorimetric method.

RESULTS

Presence of DIMBOA-glucoside in the honeydew of R. padi feeding on wheat. DIMBOA glucoside was found in the honeydew of aphids caged on all six wheat cultivars. Neither aglucone nor MBOA were found in the honeydew samples analyzed.

DIMBOA glucoside concentrations in the honeydew of aphids feeding on wheat are shown in the lower curve of figure 1. Rates of honeydew production are shown in relation to whole-leaf DIMBOA concentration in the upper curve of figure 1. Honeydew production in oats, a plant lacking Hx, was 5.35 ± 0.16 μg/aphid/36 h.

Maize leaf Hx concentration and the growth rate of R. padi. The mean Hx concentrations (± 95% confidence limits) in the seedlings of the two maize varieties used in this experiment were 1.19 ± 0.35 mmol/kg fr. wt for B37 and 4.10 ± 0.44 mmol/kg fr. wt for B49. Over the first 24 h, the M.R.G.R. of the aphids was significantly higher on B49 relative to B37 ($F= 24.11$; d.f.= 1.33; $P<0.001$; Fig. 2). This difference continued for the remaining periods, but was not statistically significant. There were no significant differences between ths variaties in terms of aphid mortality during the period studied.

DISCUSSION

R. padi is mainly a phloem feeder (Pollard, 1973). Hydroxamic acids are known to occur in mesophyll cells of wheat plants (Argandoña et al., 1987). The present finding of DIMBOA glucoside in aphid honeydew suggests that the chemicals are also present in wheat phloem. Further evidence for the presence of DIMBOA glucoside in phloem is provided by the failure to detect the aglucone in any of the honeydew samples: if prolonged ingestion had occurred from mesophyll cells ruptured during stylet penetration, then contact with ß-glucosidase would be expected and DIMBOA should have been found in the honeydew. The lack of any DIMBOA in the samples is also evidence of the absence of a suitable ß-glucosidase in the digestive tract of R. padi. The failure of Molyneux et al. (1990) to find MBOA in the

honeydew of Schizaphis graminum feeding on a DIMBOA-containing wheat is not inconsistent with these results, since the glucoside does not produce the aglucone breakdown product MBOA under the conditions they used. Neither are the results inconsistent with electronic monitoring studies of aphid feeding behaviour, which suggest the presence of hydroxamic acids in the phloem sap of the same wheat cultivars employed in this study in concentrations below those producing feeding deterrency (Givovich and Niemeyer, 1991).

The relationship between glucoside concentration present in honeydew and whole-leaf DIMBOA concentration in wheat followed a "bell-shaped" pattern (lower curve in Fig. 1). This suggests passive ingestion from phloem sap at low leaf concentrations, and a combination of feeding deterrency, possibly during stylet penetration of the mesophyll, and ingestion from tissues with lower or non-existent Hx glucoside concentrations, at high whole-leaf concentrations. Electronic monitoring of aphid feeding behavior showed that ingestion from xylem, a tissue where Hx have not been detected (Argandoña and Corcuera, 1985), increased as whole-leaf Hx concentration increased (Givovich and Niemeyer, 1991).

A "bell-shaped" curve can also be seen in the relationship between honeydew production and whole-leaf concentration of DIMBOA in the wheat cultivars (upper curve in Fig. 1). The negative correlation between honeydew production and DIMBOA concentration at the high DIMBOA range may be attributed to

feeding deterrency by DIMBOA. The positive correlation obtained at the low DIMBOA range may be attributed to a higher ingestion rate (and consequently, higher excretion rate) in order to compensate for the toxic effects of increasing Hx concentration of the ingesta. These effects may be related to the capacity of Hx to inhibit mitochondrial energy-linked reactions (Niemeyer et al., 1986) and digestive enzymes (Cuevas et al., 1990). Interestingly, honeydew production in oats, a plant lacking Hx, is comparable to that in the lower DIMBOA containing wheats.

The growth rate of R. padi was not adversely affected by the gross Hx concentration of maize leaf tissue. Aphid growth rate over the first 24 h after caging was significantly higher on B49 (high leaf Hx) relative to B37 (low leaf Hx), and remained higher for the following three days (Fig. 2). The possibility that R. padi is resistant to the toxic effects of Hx may be discounted because of the wheat results described above, although previous research had indicated that survival of a related species (R. maidis) was only slightly reduced by increasing levels of DIMBOA in an artificial diet (Corcuera et al., 1982).

One explanation for the M.R.G.R. differences is that the phloem sap of B49 may contain higher concentrations of certain amino acids, in particular those containing nucleophilic residues. These latter have been shown to react with DIMBOA (Niemeyer et al., 1982; Pérez and Niemeyer, 1989; Cuevas et al., 1990) thereby diminishing its toxic effects (Argandoña et al., 1982). A higher amino acid concentration in the phloem of B49

would thus not only mean higher nutritional value but also lower toxicity due to Hx.

Alternatively, the leaf concentration of Hx in the varieties studied may not parallel their concentration in the phloem sap; some plants do differ in their partitioning of Hx. For example, it has been recently shown that while both rye and wheat seedlings accumulate Hx in their roots, only rye exudes them through the roots (Pérez and Ormeño-Núñez, 1991).

These results from wheat and maize suggest that Hx may be playing different roles in resistance to aphids depending on where the toxin is located in the plant. A high mesophyll concentration of Hx may provide a level of feeding deterrency during stylet penetration, while a high phloem sap concentration may also provide deterrency combined with antibiosis. The deterrency and antibiosis effect of phloem Hx may conceivably be a result of a complex interaction with other factors such as phloem nutritional quality. Thus the gross leaf concentration of Hx may not be a true reflection of the overall level of resistance to aphids. This may explain the sometimes low proportion of the variation in aphid performance explained by gross Hx levels in some studies (e.g. Wratten et al., 1991). Plant resistance programmes may have to take this into consideration.

Further research should determine if DIMBOA glucoside can be found directly in phloem sap; compare the phloem concentration of the glucoside in a range of maize and wheat cultivars in order to

determine whether there is a correlation between this variable and the total leaf content of DIMBOA; and determine the amino acid concentration and composition in the two maize varieties studied.

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164

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163

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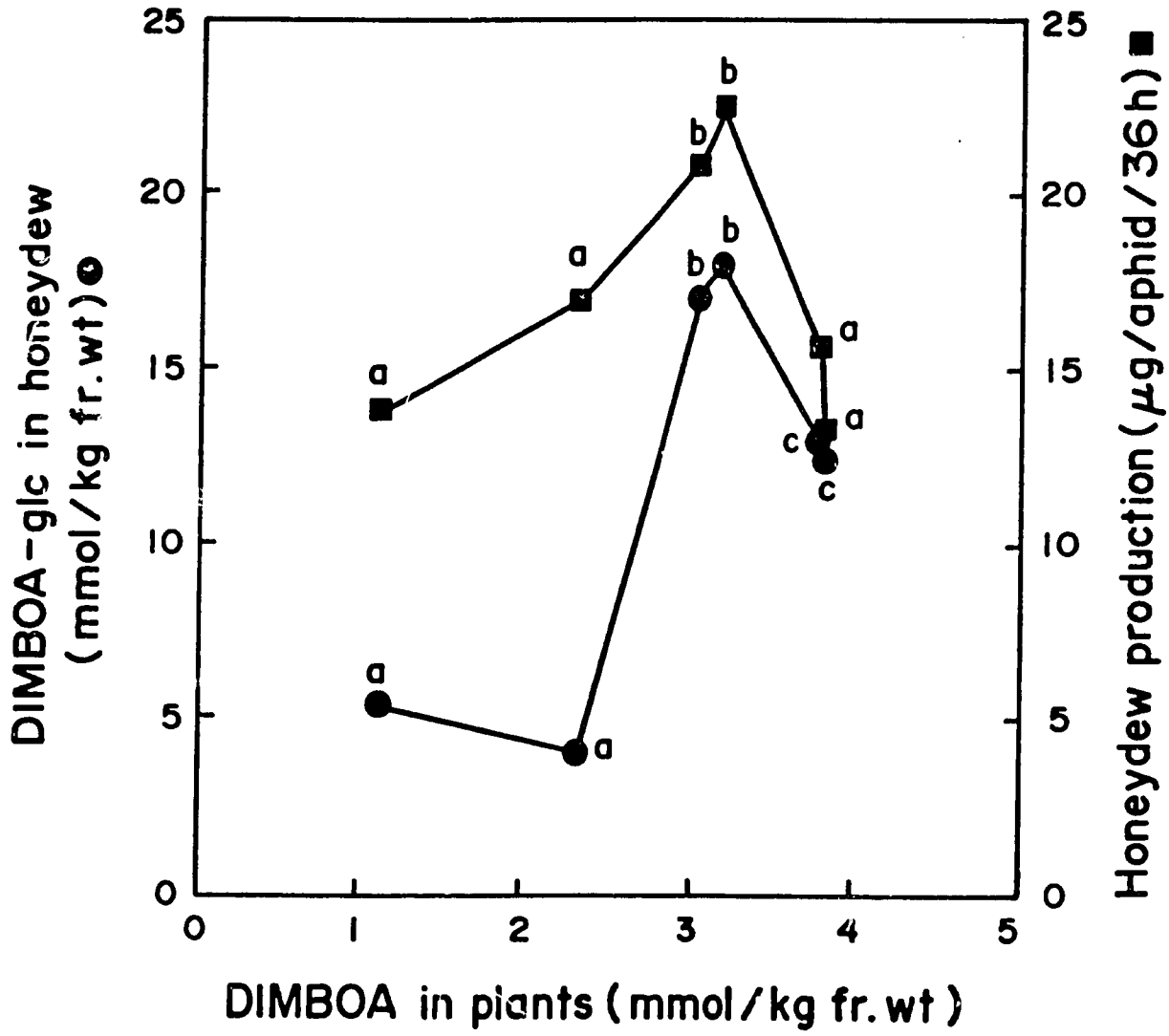
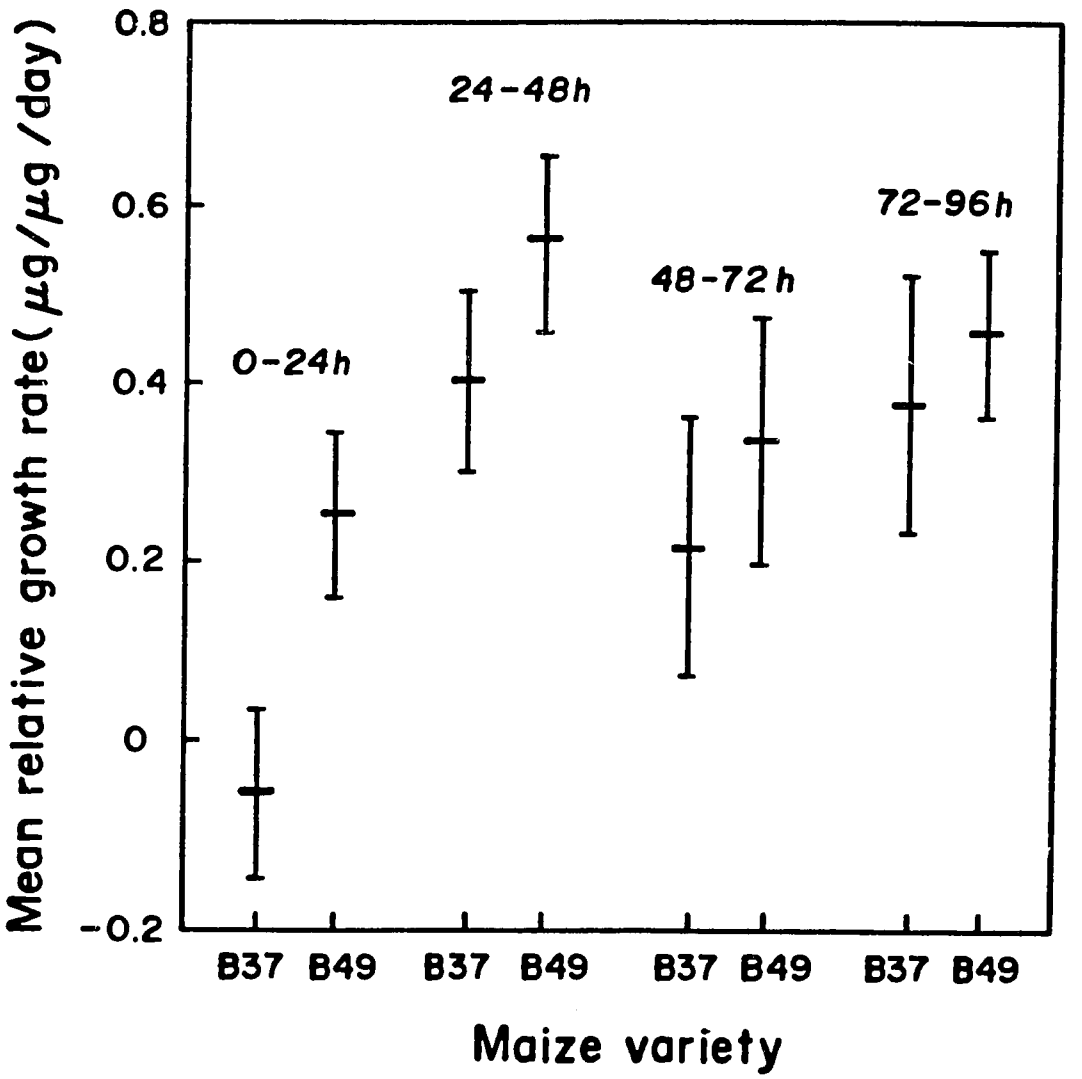
FIGURE LEGENDS

Figure 1. Mean concentration of DIMBOA glucoside in honeydew (●) and mean honeydew production (■) of R. padi, as a function of DIMBOA concentration in wheat seedlings where they fed for 36 h. Points in each curve followed by different letters are significantly different (Student t-test, $p<0.05$).

Figure 2. Mean relative growth rates (μg/μg/day, ± 95% confidence limits) for R. padi caged on two maize varieties, B37 (low leaf Hx concentration) and B49 (high leaf Hx concentration).


DIMBOA-glc in honeydew (mmol/kg fr.wt) ●
Honeydew production (µg/aphid/36h) ■
DIMBOA in plants (mmol/kg fr.wt)
0
5
10
15
20
25
0
1
2
3
4
5
a
b
c

171


Mean relative growth rate (µg/µg /day)
0.8
0.6
0.4
0.2
0
-0.2
0-24h
24-48h
48-72h
72-96h
B37
B49
B37
B49
B37
B49
B37
B49
Maize variety

172