PN-AAZ-237 3. F-03

FINAL REPORT

Grant: DPE-5542-G-4029-00

Proposal: Role of secondary metabolites in the resistance of cereals to aphids (3F-03)

Principal investigator: Hermann M. Niemeyer

OVERALL OBJECTIVES

The main objectives stated in the original proposal were to establish the role of hydroxamic acids (Hx) and simple indol alkaloids from Gramineae in plant resistance to aphids, to study environmental factors that influence the concentration of Hx in the plants and to determine the mode of action of Hx at the molecular level.

In the technical work plan, seven areas of studies were mentioned. An eighth area was considered following the provisos of the review panel. The results obtained in each area are described below.

RESULTS

a) Biological activity of compounds

Hydroxamic acids exist as glucosides in intact tissues. DIMBOA, the main Hx in wheat and maize, and its glucoside, were incorporated into artificial diets and their effects upon feeding aphids (*Schizaphis graminum*) recorded. Both compounds were toxic and acted as feeding deterrents at concentrations comparable to those found in wheat plants as a whole (Corcuera

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et al., 1985). Concentrations needed to exert the same effect were higher in the case of the glucosides. This relates Hx to numerous other plant secondary metabolites which are stored as less active glycosides.

The barley alkaloid gramine was fed to the aphids S. graminum and Rhopalosiphum padi in artificial diets. Reproductive index, survival and food ingestion were reduced by gramine concentrations comparable to those found in barley plants (Zuñiga et al., 1985; Zuñiga & Corcuera, 1986, 1988).

The barley water-stress metabolites proline and glycine-betaine were incorporated into artificial diets. Neither compound affected survival, but, while proline showed no significant effect on reproductive index, glycine-betaine increased it (Zúñiga & Corcuera, 1987a). When glycine-betaine was added to gramine-containing diets, the gramine lethal dose increased with increasing glycine-betaine concentration (Zúñiga & Corcuera, 1987b).

b) Presence of indol alkaloids and hydroxamic acids in wild and cultivated Gramineae

Wild Gramineae were screened for hydroxamic acids. Hx were found in Chusquea cumingii, Arundo donax and Elymus gayanus, but not in Bromus unioloides, Poa annua, Dactylis glomerata, Phalaris canariense, Lolium perenne, Hordeum chilense, Cynodon dactylon, Shorghum sudanense x S. vulgare and Setaria verticillata (some of these results were obtained in the period between proposal submission and approval).

Within the cultivated Gramineae, Hx were found in rye, maize and wheat, but not in barley. Additionally, Hx were found in Triticale (Corcuera et al., 1987).

Parental material for wheat breeding for high Hx levels were searched among 55 accessions belonging to 17 Triticum species. Hx were found in all of them, concentrations varying from 0.2 to 16 mmol/Kg fresh weight. Common wheats ranged from 0.7 to 5 mmol/Kg fr. wt. (Niemeyer, 1987).

Seedlings of 34 barley cultivars were screened for indol alkaloids. Gramine was the only alkaloid found, in concentrations ranging from 0 to 4.8 mmol/Kg fr. wt. (Zúñiga et al., 1985). In contradistinction, neither gramine nor hordenine were found in the following wild species: Distichlis spicata, Hordeum chilense, Lolium multiflorum, Poa australis, Bromus setifolius and Hordeum halophylum (Araya, 1987). A review on indol alkaloids from Gramineae was produced (Corcuera, 1987).

Proline, glycine-betaine and choline were found in various concentrations in these latter species (Araya, 1987).

c) Infestation experiments with aphids

Resistance to the aphid Sitobion avenae was assessed on six wheat cultivars of different Hx levels. A range of values for intrinsic rate of increase (r_m) were obtained, 96% of the variation in which was explained by Hx concentrations. Of the components of r_m, age-specific fecundity was the most significant factor contributing to resistance (Bohidar et al., 1986).

Inverse linear correlations were found between rate of growth of aphid populations in barley seedlings and their gramine content (Zúñiga et al., 1985; Zúñiga & Corcuera, 1986), and also between rate of growth of aphid populations and gramine incorporated into barley shoots (Zúñiga & Corcuera, 1988).

Water-stressed barley was more susceptible to the aphid S. graminum than barley growing under normal conditions. (Zúñiga & Corcuera, 1987a, 1987b). Additionally, incorporation of glycine-betaine into excised barley leaves increased the population growth rate of aphids (S. graminum) on the leaves. Proline incorporation showed no significant effect, but choline incorporation decreased significantly the population growth rate (Zúñiga & Corcuera, 1987a).

d) Cellular and tissular location of compounds

The distribution of Hx was examined in maize and wheat tissues. Hx were not found either in guttation drops, xylem exudate or epidermis. Both in roots and mesocotyls, Hx were more concentrated in veins than in the complete leaf (Argandoña & Corcuera, 1985; Corcuera et al., 1987; Argandoña et al., 1987).

On the other hand, gramine was not found either in guttation drops, xylem exudate or vascular bundles of barley plants. Gramine was more concentrated in epidermis than in the complete leaf (Corcuera et al., 1987; Argandoña et al., 1987).

Electric monitoring of aphid feeding behavior in a gramine-lacking barley cultivar showed that S. graminum spent more time in phloem ingestion and R. padi in non-phloem ingestion. In a gramine-containing cultivar, R. padi fed only from pnloem, and time to reach phloem increased in both aphids.

Neither aphid fed from the phloem of barley shoots to which gramine was incorporated. Additionally, R. padi ingested from non-phloem similarly

to the case of a gramine-lacking barley cultivar.

e) Factors that influence the content of Hx and indol alkaloids in plants

Water stress decreased the hydroxamic acid content of wheat seedlings (Galvez, 1986).

In barley plants, water stress did not alter significantly gramine content (Zúñiga & Corcuera, 1987b). Water-stressed barley contained significantly higher amounts of proline and glycine-betaine than barley grown under normal conditions (Zúñiga, & Corcuera, 1987a); Zúñiga & Corcuera, 1987b).

Gramine content of barley decreased with the age of the plant from the 5th day untill the 27th. In 11-day old barley seedlings, gramine levels increased with temperature in the second leaf but remained constant in the first leaf. Barley seedlings showed higher gramine content under a long photoperiod (16/8) than under a short one (8/16). Finally, nitrogen fertilization increased gramine levels in the second leaf of barley seedlings but had no effect on the first leaf (Salas, 1986).

f) Studies on the mode of action of hydroxamic acids from cereals

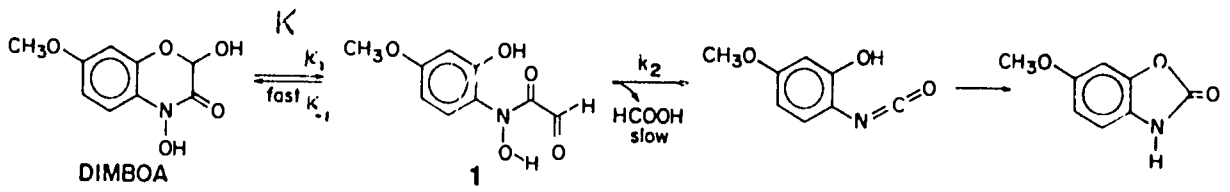
The effect of DIMBOA, the main hydroxamic acid in wheat and maize, on mitochondrial energy-linked reactions was explored as a source of its toxicity. DIMBOA inhibited reversibly ATP synthesis, P_i-ATP exchange reaction and ATPase activity in submitochondrial particles from bovine heart. At higher concentrations it also inhibited mitochondrial electron transport.

Irreversible inactivation of mitochondrial electron transport, P_i-ATP exchange reaction and 8-anilino-1-naphthalene energy-dependent fluorescent enhancement was also observed (Niemeyer et al., 1986).

A source of interaction between DIMBOA and enzymic systems are nucleophilic sites in the enzyme. DIMBOA was reduced by thiols. The reactive species were undissociated DIMBOA and thiolate anion, the mechanism proposed involving rate-limiting attack of thiolate anion on the hydroxamic nitrogen atom and fast attack of a second thiol molecule on the sulphur atom of the intermediate formed (Pérez & Niemeyer, 1985).

g) Decomposition of DIMBOA

Studies of the decomposition of DIMBOA in non-aqueous solvents supported the following mechanism:



The conclusion is based on the following data: in low donor number solvents, k_1 is rate limiting and in high donor number solvents, k_2 is rate-limiting (Bravo & Niemeyer, 1985), ii) K could be determined polarographically in high donor number solvents, and the calculated k_2 (k_{obs}/K) gave rise to a linear free solvation energy relationship with donor number (Copaja et al., 1986), iii) linear free solvation energy relationships between observed rate constants and acceptor number alcoholic solvents

(Bravo & Niemeyer, 1986b), iv) borate catalysis of the reaction in aqueous solution by formation of an adduct with compound 1 (Pérez & Niemeyer, 1986), v) a secondary product was isolated whose formation involved intermediate 1 (Bravo & Niemeyer, 1986a).

h) Field studies

Field trials were conducted on artificial infestation by *R. padi* and *Metopolophium dirhodum* of 14 wheat cultivars varying in Hx, up to day 80 after germination. Climatic factors such as rainfall or temperature could not explain variations in aphid numbers. Significant inverse correlations were found between aphid numbers and Hx in 10 cases. In the other cases, correlations were poor. Slopes and intercepts of lines varied considerably within cultivars. Slopes were smaller for *R. padi* than for *M. dirhodum*.

CONCLUSIONS

Hydroxamic acids in wheat

The scope of the correlations between aphid resistance and Hx levels in the plant was widened to include *Sitobion avenae*.

Hx inhibit various mitochondrial functions, a fact that may be related to their widespread toxicity. Chemical studies indicate that electrophilic character of the hydroxamic nitrogen atom and the (hidden) aldehyde group in Hx, is crucial for the inhibition of enzyme complexes.

Field studies indicate that although aphid infestation in wheat is related to Hx levels, other aphid-plant interaction mechanisms are also involved which vary in importance among different cultivars.

Screening of ancient and modern wheats as well as wild Gramineae has lead to the identification of suitable germplasm for breeding wheat for high Hx levels, both through interspecific and intraspecific hybridization.

Indole alkaloids in barley

Controlled infestation experiments, assays with artificial diets and electric monitoring of aphid feeding behavior indicate that gramine is involved in the protection of barley against aphid damage. The degree of protection depends on the feeding behavior of the aphid, due to the compartmentation of gramine withing the plant.

Under water stress, protection of barley against aphids decreases, due to the direct effect of water-stress metabolites and also to their interaction with gramine.

Gramine levels are influenced by age of the plant as well as by photoperiod and nitrogen fertilization.

Suggestions have been earlier put forward that gramine levels should be decreased by breeding because it is toxic to ruminants. Our results indicate that such measures might jeopardize plant survival by decreasing its defenses.

Appendix 1: Publications

Grant DPE-5542-G-SS-4029-00

Proposal: Role of secondary metabolites in the resistance of cereals to aphids (3F-03)

Principal investigator: Hermann M. Niemeyer

1. Corcuera, L.J., Queirolo, C.B. and Argandoña, V.H. (1985). Effect of 2- -D-glucosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one on Schizaphis graminum (Rondani) (Insecta, Aphididae) feeding on artificial diets, **Experientia** 41: 514-516.

2. Argandoña, V.H. and Corcuera, L.J. (1985) Distribution of hydroxamic acids in Zea mays tissues, **Phytochemistry** 24: 177-178.

3. Zuñiga, G.E., Salgado, M.S. and Corcuera, L.J. (1985) Role of an indole alkaloid in the resistance of barley seedlings to aphids, **Phytochemistry** 24: 945-947.

4. Bravo, H.R. and Niemeyer, H.M. (1985) Decomposition in aprotic solvents of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one, a hydroxamic acid from cereals, **Tetrahedron** 21: 4983-4986.

5. Pérez, F.J. and Niemeyer, H.M. (1985) The reduction of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one by thiols, **Phytochemistry** 24: 2963-2966.

6. Pérez, F.J. and Niemeyer, H.M. (1986) Effect of borate on the decomposition of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one, a hydroxamic acid from Gramineae, **Heterocycles** 24: 13-16.

7. Niemeyer, H.M., Calcaterra, N.B. and Roveri, O.A. (1986) Inhibition of mitochondrial energy-linked reactions by 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one, a hydroxamic acid from Gramineae, **Biochem. Pharmacol.** 35: 3909-3914.

8. Bravo, H.R. and Niemeyer, H.M. (1986a) A new product from the decomposition of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one, a hydroxamic acid from cereals, **Heterocycles** 24: 335-337.

9. Zúñiga, G.E. and Corcuera, L.J. (1986) Effect of gramine in the resistance of barley seedlings to the aphid Rhopalosiphum padi, **Ent. Exper. Appl.** 40: 259-262.

10. Bohidar, K., Wratten, S.D. and Niemeyer, H.M. (1986) Effects of hydroxamic acids on the resistance of wheat to the aphid Sitobion avenae, **Ann. Appl. Biol.**, 109: 193-198.

11. Copaja, S.V., Bravo, H.R. and Niemeyer, H.M. (1986) Quantitation of N-(2-hydroxy-4-methoxy-phenyl)-glyoxylo-hydroxamic acid, a reactive intermediate in reactions of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one, J. Org. Chem. 51: 3542-3545.

12. Bravo, H.R. and Niemeyer, H.M. (1986b) Interaction of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) with alcoholic solvents. **Heterocycles** 24: 2809-2812.

13. Corcuera, L.J., Argandoña, V.H. and Zúñiga, G.E. (1987) Resistance of cereal crops to aphids: role of allelochemicals. In "**Allelochemicals: Role in Agriculture, Forestry and Ecology**". Waller, G.R., ed., American Chemical Society Symposium Series, pp. 129-135.

14. Zúñiga, G.E. and Corcuera, L.J. (1987a) Glycine-betaine accumulation influences susceptibility of water stressed barley to the aphid Schizaphis graminum, **Phytochemistry** 26: 367-369.

15. Niemeyer, H.M., Calcaterra, N.B. and Roveri, O.A. (1987) Inhibition of energy metabolism by benzoxazolin-2-one, **Comp. Biochem. Physiol.**, in press.

16. Zúñiga, G.E. and Corcuera, L.J. (1987b) Glycine-betaine in water stressed barley reduced the effects of gramine on the aphids Schizaphis graminum and Rhopalosiphum padi, **Phytochemistry**, in press.

17. Argandoña, V.H., Zúñiga, G.E. & Corcuera, L.J. (1987) Distribution of gramine and hydroxamic acids in tissues of barley and wheat leaves, **Phytochemistry**, in press.

18. Niemeyer, H.M. (1987) Hydroxamic acid content of Triticum species, **Euphytica**, in press.

19. Corcuera, L.J. (1987) Indole alkaloids from Phalaris and other Gramineae. In "**Toxicants of Plant Origin. Vol. I. The Alkaloids**". Cheeke, P.R., ed., CRC Press Inc., Boca Raton, FI, in press.

20. Galvez, R., Quiroz, C. and Argandoña, V.H. (1988) Presencia de ácidos hidroxámicos en trigos cultivados en el campo y niveles de infestación de áfidos, manuscript in preparation.

21. Zúñiga, G.E. and Corcuera, L.J. (1988) Effects of gramine on the feeding behavior of the aphids Schizaphis graminum (Rondani) and Rhopalosiphum padi (L), manuscript in preparation.

Appendix 2: Communications presented in meetings

Grant DPE-5542-G-SS-4029-00

Proposal: Role of secondary metabolites in the resistance of cereals to aphids (3F-03)

Principal investigator: Hermann M. Niemeyer

A. International Meetings

1. Zuñiga, G.E. & Corcuera, L.J. (1985) Effect of betaine on susceptibility of water stressed barley to aphids. **Annual Meeting of the American Association of Plant Physiologists.** Providence, RI, USA, June 23-28 (abstract only).

2. Corcuera, L.J., Argandoña, V.H. & Zuñiga, G.E. (1985) Role of allelochemicals in the resistance of cereal crops to aphids. **190th National Meeting of the American Chemical Society**, Chicago, USA, September 8-13.

3. Niemeyer, H.M., Pérez, F.J. & Bravo, H.R. (1986) Hydroxamic acids from Gramineae. Function in the plant and mode of action. **V Conferencia de Físico-Química Orgánica**, Florianópolis, Brasil, April 2-4.

4. Corcuera, L.J. (1986) Efecto de productos naturales en la resistencia de plantas a insectos. **Reuniao de Sistematica, Evolucao e Ecologia Micromoleculares.** Rio de Janeiro, Brasil, April 28-30.

5. Argandoña, V.H. & Corcuera, L.J. (1986) Distribution of gramine in barley tissues. **Annual Meeting of the American Association of Plant Physiologists.** Baton Rouge, Louisiana, USA, June 8-12 (abstract only).

6. Niemeyer, H.M. & Pérez, F.J. (1986) Hydroxamic acids from Gramineae: their role in aphid resistance and their mode of action. **Sixth International Symposium on Insect-Plant Relationships**, Pau, France, July 1-5.

7. Wratten, S.D., Niemeyer, H.M., Thackray, D.J. & Edwards, P.J. (1986) Effects of hydroxamic acids on the resistance of wheat to the aphid Sitobion avenae. **Sixth International Symposium on Insect-Plant Relationships**, Pau, France, July 1-5.

8. Niemeyer, H.M. y Pérez, F.J. (1986) Los ácidos hidroxámicos del trigo y la obtención de variedades resistentes a áfidos. **XVII Congreso Latinoamericano de Química**, Bogotá, Colombia, October 12-19.

B. National Meetings

1. Zúñiga, G.E. & Corcuera, L.J. (1984) Papel de un alcaloide indólico en la resistencia de la cebada a áfidos. **Reunión Nacional de Botánica**, La Serena, September 24-29.

2. Zúñiga, G.E. & Corcuera, L.J. (1984) Papel de la gramina en la resistencia de la cebada a Schizaphis graminum. **Jornadas Agronómicas**, Santiago, October 1-5.

3. Zúñiga, G.E. & Corcuera, L.J. (1984) Efecto de algunos alcaloides indólicos simples en la resistencia de la cebada a áfidos. **Reunión Nacional de Productos Naturales**, Valdivia, November 13-16.

4. Argandoña, V.H. (1985) Localización tisular de metabolitos secundarios en gramíneas y tasa de crecimiento poblacional de Schizaphis graminum. **Reunión Anual de la Sociedad de Biología de Chile**, Pucón, November 28-30.

5. Zúñiga, G.E. & Corcuera, L.J. (1985) Efectos de la gramina en la resistencia de la cebada a los áfidos Schizaphis graminum y Rhopalosiphum padi. **Reunión Anual de la Sociedad de Biología de Chile**, Pucón, November 28-30.

6. Niemeyer, H.M., Pérez, F.J. & Bravo, H.R. (1985) Acidos hidroxámicos de gramíneas: función en la planta y modo de acción. **Simposio de Bioorgánica**, Concepción, November 26-29.

7. Bravo, H.R. & Niemeyer, H.M. (1985) Un nuevo producto de la descomposición de la 2,4-dihidroxi-7-metoxi-benzoxazin-3-ona (DIMBOA), un ácido hidroxámico de gramíneas. **Jornadas Chilenas de Química**, Osorno, December 3-6.

8. Pérez, F.J. & Niemeyer, H.M. (1985) Efectos micelares sobre el equilibrio ácido-base y la velocidad de descomposición del DIMBOA. **Jornadas Chilenas de Química**, Osorno, December 3-6.

9. Sepúlveda, B.A. & Corcuera, L.J. (1985) Posible papel aleloquímico de un alcaloide indólico de la cebada sobre Heliothis zea y Pseudomonas syringae, **Jornadas Chilenas de Química**, Osorno, December 3-6.

10. Zúñiga, G.E. & Corcuera, L.J. (1985) Efectos aleloquímicos inducidos por el déficit hídrico en la cebada. **Jornadas Chilenas de Química**, Osorno, December 3-6.

11. Niemeyer, H.M. y Pérez, F.J. (1986) Acidos hidroxámicos en trigo: un criterio químico para la selección de variedades resistentes a áfidos. **Simposio de Química y Utilización de Recursos Naturales**, Concepción, July 22-25.

12. Zúñiga, G.E., Sepúlveda, B.A. & Corcuera, L.J. (1986) Inhibición de los efectos de gramina por glicina-betaína en Pseudomonas syringae. **Reunión Nacional de Botánica**, Valdivia, September 23-26.

13. Gálvez, G.R. & Argandoña, V.H. (1986) Resistencia química a áfidos en trigos cultivados en el campo. **Reunión Nacional de Botánica**, Valdivia, September 23-26.

14. Zúñiga, G.E., Argandoña, V.H. & Corcuera, L.J. (1986) Distribución de prolina y glicina-betaína en cebada. **Reunión Nacional de Botánica**, Valdivia, September 23-26.

16. Sepúlveda, B.A. & Corcuera, L.J. (1986) Efecto y mecanismo de acción de la gramina sobre Pseudomonas syringae. **Reunión Nacional de Botánica**, Valdivia, September 23-26.

17. Zúñiga, G.E. & Corcuera, L.J. (1986) Efecto de gramina sobre la distribución de áfidos en cebada. **Reunión Nacional de Botánica**, Valdivia, September 23-26.

18. Salas, M.L. & Corcuera, L.J. (1986) Efecto del nitrato de potasio sobre la concentración de gramina y la resistencia de la cebada al áfido Schizaphis graminum. **Reunión Nacional de Botánica**, Valdivia, September 23-26.

19. Araya, F. & Corcuera, L.J. (1986) Efecto de iones en la resistencia de Distichlis spicata al áfido Schizaphis graminum. **Reunión Nacional de Botánica**, Valdivia, September 23-26.

20. Pérez, F.J. & Niemeyer, H.M. (1986) Inactivación de la papaína por 1,4-benzoxazinonas. **Reunión de la Sociedad de Biología de Chile**, Pucón, November 26-29.

Appendix 3: Student training

Grant DPE-5542-G-SS-4029-00

Proposal: Role of secondary metabolites in the resistance of cereals to aphids (3F-03)

Principal investigator: Hermann M. Niemeyer

A. Thesis (finished)

1. Gálvez, R.H. (1986) Presencia de ácidos hidroxámicos en trigos cultivados en el campo y niveles de infestación de áfidos. Thesis for Licenciado in Education (Chemistry and Biology), Facultad de Ciencia, Universidad de Santiago de Chile.

2. Salas, M.L. (1986) Factores ambientales y fisiológicos que afectan el contenido de gramina en cebada. Thesis for Licenciado in Education (Chemistry and Biology), Facultad de Ciencia, Universidad de Santiago de Chile.

3. Araya, F. (1987) Salinidad y resistencia de gramíneas cultivadas y silvestres al ataque de áfidos. Thesis for M.Sc. in Biological Sciences, Universidad de Chile.

4. Zúñiga, G.E. (1987) Papel de los alcaloides indólicos en la resistencia de la cebada a áfidos. Thesis for M.Sc. in Biological Sciences, Universidad de Chile.

B. Thesis (in progress)

1. Pérez, F.J. (1987) Interpretación bioquímica de la actividad biológica de los ácidos hidroxámicos de gramíneas. Thesis for Ph.D. in Biological Sciences, Universidad de Chile.

2. Sepúlveda, B.A. (1987) Papel de metabolitos secundarios de cereales en la protección natural contra el ataque de *Heliothis zea* y *Pseudomonas syringae*. Thesis for Ph.D. in Biological Sciences, Universidad de Chile.

C. Recently incorporated students

1. Givovich, A. (Insect-Plant Interactions)

2. Massardo, F. (Plant Physiology)

3. Quiroz, A. (Organic Reaction Mechanisms)

Appendix 4: Financial report

Grant: DPE-5542-G-SS-4029-00

Proposal: role of secondary metabolites in the resistance of cereals to aphids (3F-03)

Principal investigator: Hermann M. Niemeyer

Equipment

Refrigerated centrifuge (Sorvall RC-5B)	US$ 17.833
Gas chromatograph (Shimadzu GC-9A)	
Liquid Chromatograph (Shimadzu LC-6A)	
Spectrophotometers (Shimadzu UV-240 and UV-120)	43,677
Electronic balance (Shimadzu EB-2800)	
Personal computer (IBM PC-AT)	6,460
Refrigerated baths (Lauda RM6 and RM20)	4,364
Recorder (Graphtech SR-6211)	1,530
Microscopes (Nikon XF-21E and SMZ-2T-D)	9,504
	US$ 83,368

Supplies

Labware (VWR, Millipore, Hellma, Electrothermal)	US$ 15,125.04
Glassware (Kontes, Aceglass, IVA	6,093.00
Chemicals (Sigma, Fluka)	1,544.38
	US$ 22,762.42

Salaries

Graduate students:

Arturo Givovich	US$ 1,410.31
Antonieta Oehrens	795.35
Antonio Sepúlveda	2,150.66
Gustavo Zúñiga	2,583.74

Undergraduate students:

Ricardo Gálvez	467.44
Victoria Rojas	677.00
Luz Salas	795.35

Technicians:

Vivian Benavides	1,537.28
Jacqueline Grez	581.96
Lilian Muñoz	2,612.49
	US$ 13,611.58

Travel

In 1985 (Luis J. Corcuera, USA)	US$ 2,175
In 1987 (Hermann M. Niemeyer, USA and Canada)	US$ 2,809
	US$ 4.984

Other costs

Interfaces and software (Data Translation)	US$ 5,274

SUMMARY

	Requested	Spent
Equipment	US$ 82,200	US$ 83,368.00
Supplies	21,000	22,762.42
Salaries	12,000	13,611.58
Travel	9,500	4,984.00
Publications costs	800	---
Other costs	4,500	5,274.00
TOTAL	US$130,000	US$130,000.00

Hermann M. Niemeyer
Principal investigator

Camilo Quezada
Dean

Experientia 41 (1985), Birkhauser Verlag, CH 4010 Basel/Switzerland

Effects of 2-β-D-glucosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one on *Schizaphis graminum* (Rondani) (Insecta, Aphididae) feeding on artificial diets

L.J. Corcuera[1], C.B. Queirolo and V.H. Argandoña

Departamento de Biologia, Facultad de Ciencias Básicas y Farmacéuticas, Universidad de Chile, Casilla 653, Santiago (Chile),
29 May 1984

Summary. 2-β-Glucosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA-Glc), the main hydroxamic acid from maize and wheat, and its aglucone, decreased survival of *Schizaphis graminum* reared on artificial diets. Both compounds were toxic for aphids and acted as feeding deterrents, at concentrations as low as 1 mM. The natural concentrations of glucosides of hydroxamic acids in wheat leaves reach up to 6 mmoles/kg fresh weight, thus falling within the range in which DIMBOA-Glc causes deleterious effects to diet-fed aphids.
Key words. Hydroxamic acid; cereals; maize, wheat; aphids; greenbug.

Hydroxamic acids from Poaceae (Gramineae) seem to play a role in plant resistance to *Ostrinia nubilalis*[1], *Rhopalosiphum maidis*[2], *Metopolophium dirhodum*[4] and *Schizaphis graminum*[5]. DIMBOA, the main hydroxamic acid from maize and wheat extracts, decreases feeding, survival and reproduction rate of aphids reared on artificial diets[6,7]. However, DIMBOA is present in tissues as a glucoside (DIMBOA-Glc). The purpose of this work is to describe the effects on aphids of DIMBOA-Glc and to compare them with those of DIMBOA.

Experimental. Isolation of DIMBOA-Glc. Seedlings (*Zea mays* L. cv T129s) were grown in a greenhouse under permanent light at 30°C. 7-day-old seedlings (950 g) were slowly added to 1000 ml of boiling water, keeping the temperature above 90°C. After 15 min the seedlings were cooled down, homogenized and filtered through cheesecloth. The pH of the extracts was adjusted to 10 and the sample centrifuged at 10,000 × g for 10 min. The supernatant fluid was acidified (pH 3, HCl) and washed three times with diethylether (2:1 v/v ether:extract). The volume of the aqueous phase was reduced to 25 ml and added to 600 ml of methanol. The resulting suspension was filtered and the solid (22.9 g) discarded. The volume of the filtrate was reduced to 20 ml to which 100 ml of methanol were added. The solid formed (1.2 g) was removed by filtration and discarded. Acetone (300 ml) was added to the solution and the precipitate formed (17.6 g) was also removed by filtration. The solution was evaporated to dryness and dissolved in 50 ml of water:methanol 3:1 v/v.

This solution was added to a column (300 × 35 mm i.d.) of SP-Sephadex C-25 (Pharmacia) in the Fe(III) form and equilibrated with water:methanol 3:1 v/v. This column binds hydroxamic acids, turning a deep blue[8]. The column was washed with 1700 ml of the same solvent (33 ml/h). The sample was then eluted with 500 ml of water:methanol 3:1 v/v saturated with NaCl. The blue fractions were collected (158 ml). The iron was displaced from its hydroxamic acid complex by addition of 70 ml of 0.83 M EDTA pH 8.5. The precipitate formed was removed by filtration. The solution was neutralized with NaOH, concentrated and filtered. Aliquots of 6.5 ml were added to a Sephadex G-10 column (720 × 45 mm i.d.) previously equilibrated with $CHCl_3$-saturated water and eluted with the same solvent as described[9], with a flow rate of 13 ml/h, collecting 2-ml fractions.

Elution profiles were made by measuring the absorbance of the fractions at 260 nm, by treating the fractions with $FeCl_3$ and

measuring the absorbance at 590 nm, and by treating the samples with anthrone reagent for sugars and measuring the absorbance at 620 nm[10]. Only one peak which contained both hydroxamic acid and sugar was observed. This peak had an elution volume of 615 ml. Peaks with elution volumes of 424 and 475 ml co-chromatographed with Fe-EDTA complexes. The $FeCl_3$ and anthrone positive fractions were pooled and evaporated to dryness. The compound obtained (m.p. 250-251 °C) had equimolar amounts of hydroxamic acid and sugar, as determined by the $FeCl_3$ and anthrone tests, respectively. The compound was identified as DIMBOA-Glc by its UV, NMR and mass spectra, which were the same as those published[11,12]. The yield of DIMBOA-Glc was 1.3 g/kg of fresh tissue. DIMBOA was isolated as described[13].

Aphid assays. Individuals of *Schizaphis graminum* (Rondani) were collected from naturally-infested barley near Santiago and allowed to reproduce on plants of *Hordeum distichum* L. cv Fola Union kept under continuous light at room temperature. Feeding assays were made with diets placed between two layers of Parafilm M[14]. The diet was as described[15], plus DIMBOA-Glc or DIMBOA. When young aphids were used for experiments, they were 3rd and 4th instar nymphs. All experiments were performed at 28 °C under continuous light. SE were less than 10% and were omitted from the figures for simplicity.

Results. Survival and reproduction of aphids. DIMBOA-Glc and DIMBOA were offered separately to nymphs of *S. graminum* in diets (fig. 1). Both compounds decreased survival of aphids, DIMBOA being more lethal than DIMBOA-Glc. The concentrations necessary to produce 50% mortality were 4.0 and 1.2 mM DIMBOA-Glc and DIMBOA, respectively.

Adults of *S. graminum* were fed DIMBOA-Glc or DIMBOA and reproduction rates were measured (fig. 2). Both compounds decreased reproduction rate at concentrations as low as 0.25 mM.

Toxicity and feeding deterrence. Aphid nymphs of *S. graminum* were exposed to diets containing DIMBOA-Glc or DIMBOA and the fraction of aphids that were feeding on the diets was determined. Both compounds showed appreciable feeding deterrent activity even at the lowest concentration tested (0.5 mM).

Cohorts of nymphs of *S. graminum* were offered diets containing DIMBOA-Glc or DIMBOA and then transferred to diets without hydroxamic acids. Aphid survival was determined (fig. 3). The lowest survivals were observed with 6 mM DIMBOA-Glc and 4 mM DIMBOA. At higher concentrations both DIMBOA-Glc and DIMBOA have a strong feeding deterrent activity. Hence, ingestion of the diets with the compounds was limited and survival was higher. At intermediate concentrations more compound was ingested causing a lower survival of the aphids. Thus, DIMBOA-Glc and DIMBOA have both feeding deterrent and toxic activities on *S. graminum* feeding on artificial diets.

Discussion. Most of the knowledge about the activity of hydroxamic acids from maize and wheat on insects has been obtained by testing the effects of DIMBOA[2-7]. Since DIMBOA is

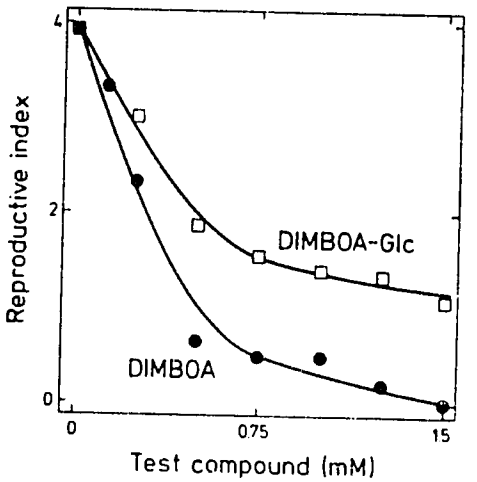


Figure 2. Effect of DIMBOA-Glc and DIMBOA on reproduction of adults of *S. graminum* reared on artificial diets. Reproductive index (number of nymphs born/number of adults alive) was determined after feeding adults for 50 h. Each point is the mean of five samples of five adults each.

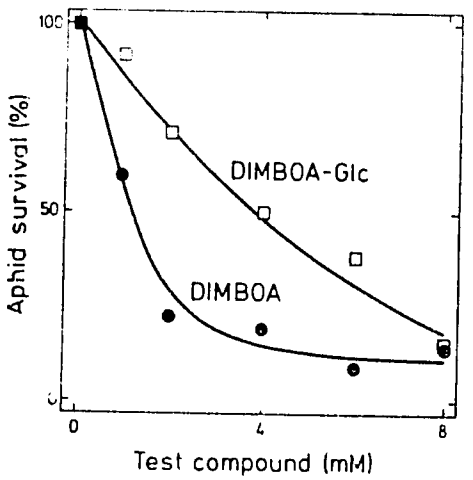


Figure 1. Effect of DIMBOA-Glc and DIMBOA on survival of nymphs of *S. graminum* reared on artificial diets. Survival, expressed as percent of initial individuals, was determined after 24 h of feeding. Each point is the average of three samples of 10 aphids each.

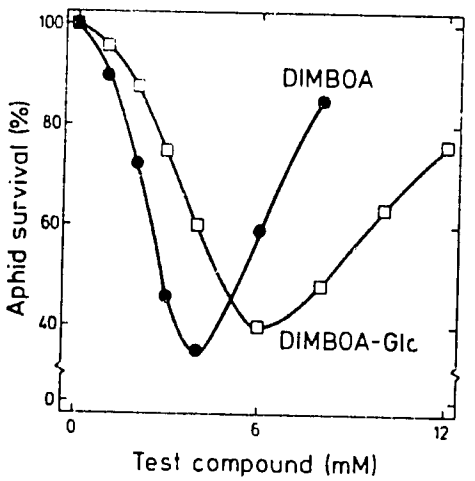


Figure 3. Effect on aphid survival of exposure for a limited time to DIMBOA-Glc or DIMBOA. Nymphs of *S. graminum* were exposed to diets with DIMBOA-Glc or DIMBOA for 12 h and then transferred to control diets without hydroxamic acids. Survival was determined 24 h later as a function of hydroxamic acid concentration in the initial diets. Each point is the average of three samples initially of 10 aphids each.

not found in intact tissues, the properties of the naturally-present DIMBOA-Glc may be important in plant-insect interactions. Although DIMBOA was always a more active molecule, DIMBOA-Glc also showed feeding deterrent and toxic activities on *S. graminum*. Since DIMBOA-Glc may be hydrolyzed to DIMBOA upon disruption of cells during insect feeding[11], the toxic and feeding deterrent properties of both compounds may be relevant to plant protection. The effects of both compounds on aphids fed on artificial diets were observed at a concentration range similar to that found in wheat leaves (0.1 to 6.3 mmoles/kg of fresh weight). Since an inverse correlation between hydroxamic acid content of leaves and aphid infestation level has been found[4,5], it is possible that varieties resistant to aphids may be obtained by selecting those with higher hydroxamic acid content. It is difficult to predict the concentration of hydroxamic acid necessary to achieve plant protection against *S. graminum*. However, it has been found that plants with more than 4 mmoles/kg of fresh weight in their leaves are not susceptible to *S. graminum* and *Metopolophium dirhodum*[4,6].

1 To whom correspondence should be addressed. This work was supported by grants from International Foundation for Science (484), Universidad de Chile (N-1654) and the Agency for International Development.

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DISTRIBUTION OF HYDROXAMIC ACIDS IN *ZEA MAYS* TISSUES

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(*Revised received* 7 *June* 1984)

Key Word Index—*Zea mays*; maize; *Triticum durum*; wheat; Gramineae; 1,4-benzoxazin-3-ones.

Abstract—The hydroxamic acid content of leaves of cereals correlates well with resistance to aphids. In maize these compounds were absent from xylem exudates and guttation drops. Lateral veins of leaves of 7-day-old maize plants contained 8 mmol/kg fr. wt while the entire leaf contained only 4.2 mmol/kg fr. wt. In leaves of 20-day-old plants, these amounts decreased by *ca* one-third. In mesocotyls, the cortex and central vascular cylinder contained 1.3 and 2.2 mmol/kg fr. wt, respectively. In 12-day-old wheat plants, the complete leaves and their veins contained 2.4 and 6.4 mmol/kg fr. wt respectively. Thus, the concentration of hydroxamic acid was always higher in the vascular bundles.

INTRODUCTION

Plant secondary metabolites may be important in protecting plants against herbivores and plant pathogens. For example, hydroxamic acids from the Gramineae have been suggested as resistance factors to several insects [1–3]. DIMBOA, the main hydroxamic acid from maize and wheat extracts, is toxic and deters feeding in the greenbug *Schizaphis graminum* (Rondani) at concentrations lower than those usually found in plant sap [4]. Hydroxamic acids are found in roots, shoots and leaves of maize but their location in the tissues of these organs is unknown [5]. Secondary metabolites, such as cyanogenic glucosides in *Sorghum*, may be compartmentalized in plant tissues [6]. In this paper we report that in maize hydroxamic acids are preferentially concentrated around vascular tissues.

RESULTS AND DISCUSSION

Hydroxamic acids were not homogeneously distributed among the various parts of maize leaves of 7- and 20-day-old plants (Table 1). The concentration in the lateral veins was higher than that in the rest of the leaf. Additionally, hydroxamic acids were not detected in guttation drops or xylem exudates. Similar results were obtained when several leaves of a 35-day-old plant were analysed (Table 2). The concentration was always higher in lateral veins than in the complete leaf. This was also observed in leaves of a 12-day-old plant of *Triticum durum* cv SNA-3. The concentration of hydroxamic acid in the vascular tissues of these leaves was 6.4 mmol/kg fr. wt while the concentration in the complete leaf was only 2.4.

Hydroxamic acids were present in mesocotyls and roots (Table 3) and, as previously reported [3], their concentration decreased as plants became older. Also, hydroxamic acids were more concentrated in the stele (vascular cylinder) than in the cortex.

It has been proposed that hydroxamic acids may play a role in iron transport because of their high affinity for this element [7, 8]. The absence of these compounds from xylem exudates and guttation drops, as well as the high concentration of organic acids in xylem exudates [9], suggests that hydroxamic acids are not important in transport of metals through to xylem. However, they could participate in plant mineral nutrition in other plant compartments.

The high concentration of hydroxamic acids observed in stele tissue of leaves of maize and wheat may be of importance for the resistance of the plants to aphids. The greenbug *Schizaphis graminum* feeds predominantly from the phloem and secondarily from mesophyll tissue of its host plant [10]. Phloem and other tissues found in the stele may be protected by the high concentration of hydroxamic acids against aphid feeding because of their toxic and feeding deterrent properties [4].

Table 1. Hydroxamic acid distribution in leaves of maize

Leaf part*	Hydroxamic acid (mmol/kg fr. wt) 7-Day-old plants	20-Day-old plants
Complete leaf	4.2 ± 0.1	1.3 ± 0.1
Central vein	4.8 ± 0.1	1.0 ± 0.1
Lateral veins	8.0 ± 0.2	2.7 ± 0.2
Guttation drops	n.d.	n.d
Xylem exudate	n.d.	n.d

* Plants were grown in a greenhouse. The second leaf to appear was used for analyses. Guttation drops were obtained from the tips of leaves. Xylem exudates from leaves were obtained by making a transverse section of the leaves and collecting drops that accumulated on the leaves attached to the plants. The numbers (± s.e.) are the mean of two samples of several leaves each. n.d. = not detected.

Table 2. Hydroxamic acid content in various leaves of a maize plant

	Hydroxamic acid (mmol/kg fr. wt)		
Leaf*	Central vein	Lateral vein	Complete leaf
Second	0.6 ± 0.1	0.9 ± 0.0	0.8 ± 0.1
Third	1.0 ± 0.1	3.2 ± 0.2	0.9 ± 0.1
Fourth	1.9 ± 0.2	4.8 ± 0.3	1.9 ± 0.2

* Plants were grown in a greenhouse for 35 days. Leaves were ordered from the oldest (first to appear) to the youngest. The first leaf was not analysed because of its senescent state. The numbers (± s.e.) are the mean of two samples of several equivalent leaves of various plants.

Table 3. Hydroxamic acid content in cortex and vascular cylinder of roots and mesocotyls of maize seedlings

	Hydroxamic acid* (mmol/kg fr. wt)					
	Root		Mesocotyl			
Plant part	4†	8	4†	6	7	8
Cortex	3.1	1.0	3.6	5.9	1.1	1.3
Vascular cylinder	4.5	2.3	7.5	6.1	3.1	2.2
Complete organ	3.5	1.5	5.7	6.2	1.9	1.4

* Plants of maize were grown in the dark at 28. Values represent the mean of two samples. Standard errors were similar to those recorded in Tables 1 and 2 and are omitted for the sake of simplicity.

† Plant age (days).

EXPERIMENTAL

Plant tissue. Plants of *Zea mays* cv. T129s and *Triticum durum* cv. SNA-3 were grown in soil in a greenhouse under permanent light, being irrigated with tap H_2O. Temp. varied between 22° at night and 28° during the day. Plant tissues were homogenized in distilled H_2O and centrifuged at 3500 g for 15 min. The supernatant fluid was adjusted to pH 3 (1 M HCl), extracted into Et_2O (2:1 v/v, Et_2O-extract) and evapd to dryness. This extract was used for quantitation of hydroxamic acids as previously described [3].

Separation of tissues. Primary and secondary veins from leaves were separated using carborundum as described in ref. [11] and then by mechanical separation with a dissection needle under a stereoscope. Cortex and stele from dark-grown plants were separated by twisting the mesocotyl at its base to break the cortical cylinder but not the stele [12]. The stele was then pulled out from the base of the mesocotyl. The separated cortical and vascular cylinders were used for analyses of hydroxamic acids. Vascular cylinders from principal roots were obtained by making a longitudinal cut 3–5 mm below the transition zone which allowed the removal of the cortex.

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ROLE OF AN INDOLE ALKALOID IN THE RESISTANCE OF BARLEY SEEDLINGS TO APHIDS

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(*Revised received* 10 *September* 1984)

Key Word Index *Hordeum*; Gramineae; *Schizaphis graminum*; greenbug; gramine; benzyl alcohol

Abstract—The content of the simple indole alkaloid gramine in barley leaves decreased with age. Conversely, susceptibility to aphids increased in older plants. Population growth rate of the greenbug *Schizaphis graminum* correlated with gramine content of leaves of several barley cultivars. Gramine decreased rate of feeding, survival and reproductive index of aphids feeding on artificial diets at concentrations similar to those found in plant leaves. Thus, it is suggested that gramine plays a role in the resistance of barley seedlings to *S. graminum*. Benzyl alcohol, a previously reported insect resistance factor from barley, was absent from all barley cultivars analysed.

INTRODUCTION

Plant chemicals may affect resistance of several Gramineae to aphids. Resistance of *Sorghum* to the aphid *Schizaphis graminum* has been associated to the degree of methylation of the intercellular pectin [1]. Hydroxamic acid content in leaves of corn, rye and wheat correlates with plant resistance to aphids [2–4]. It has been proposed that resistance of some barley varieties may be attributable to the presence of several phenolics or flavonoids [5]. Benzyl alcohol also has been suggested as a possible resistance factor of small grains to the greenbug [6], but these results have not been confirmed.

Gramine (I), a simple indole alkaloid, is present in several barley cultivars reaching concentrations of up to 8 mg/g dry wt [7–9]. Indole alkaloids are responsible for toxicity of *Phalaris* pastures to sheep and cattle [10–12]. These compounds cause various effects on ruminants [13], decrease food palatability to lambs [14], and cause kidney lesions, glycosuria and weight losses in meadow voles fed on gramine [15]. Indole alkaloids also decrease survival of aphids feeding on artificial diets and are feeding deterrents [16]. In this paper, the possible roles of benzyl alcohol and gramine in the resistance of barley to *S. graminum* are discussed.

RESULTS

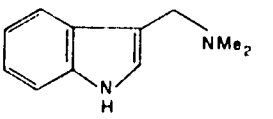
Benzyl alcohol and gramine in barley

Benzyl alcohol, a previously reported plant resistance factor to *S. graminum* [6], was not detected in any of the barley cultivars analysed (cv. Rogers, Omugi, F. Union, Cruzat and Australiana). Plants analysed were 10-day-old, grown in the dark, or 20–30-day-old, grown under light at 25°. The limit of detection of the method was 0.01 mmol/kg fr.wt. Since a much higher concentration of benzyl alcohol is required to cause deleterious effects on *S. graminum* feeding on artificial diets (LD_{50} 0.3 mM) it may be suggested that this compound is not a resistance factor of barley to aphids.

Seeds of 34 barley cultivars were germinated in the dark, at 25° for 6 days, and the gramine content of the shoots (mesocotyl and coleoptile) was determined by using Ehmann's reagent for indoles (see Experimental). Each extract was also analysed by TLC, gramine being the only indole alkaloid present. Cultivars differ in their gramine content, ranging from 0 to 4.8 mmol/kg fr.wt. Gramine was not detected in seeds. Cultivars Rogers and Omugi had a gramine content of 0.25 and 0.7 mmol/kg fr.wt, respectively.

Gramine content and resistance to aphids

Varieties that lack benzyl alcohol and differ in gramine content were selected for aphid infestation experiments. Ten-day-old plants were infested with aphids and the insect population and gramine content of leaves were determined at 10 and 20 days (Fig. 1). Gramine content decreased with plant age. The most susceptible variety was F. Union, a gramine-lacking plant, while varieties with a higher gramine content than average were more resistant to the aphids. Older plants were more susceptible to aphids (not shown). To explore further this apparent effect of gramine on aphid population growth rate, several barley varieties were infested with *S. graminum* and the population growth rate was determined over a 6-day interval. A correlation ($r = -0.98$) was observed between gramine content and aphid population growth rate (Fig. 2).



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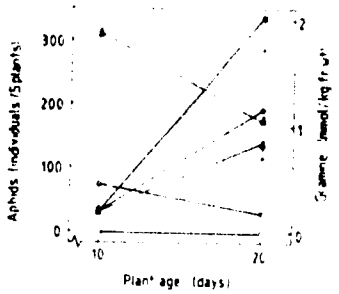

Fig. 1. Gramine content and susceptibility of barley leaves to *Schizaphis graminum*. Filled and open symbols indicate aphid population and gramine content, respectively. (▲ ---, barley cv. Cruzat; ● —, cv. Australiana; ■ ---, cv. F. Union). Two nonalate adults were placed on each 10-day-old plant. Values are the average of three samples of five plants each. Vertical bars are 1 s.e.

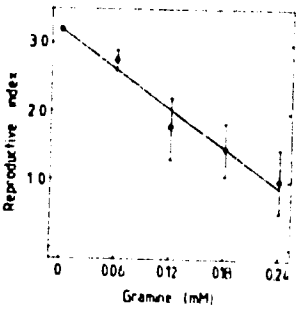

Fig. 4. Effect of gramine on reproduction of *Schizaphis graminum* reared with artificial diet. The reproductive index (number of nymphs/average number of adults) was measured after feeding aphid adults for 72 hr. Each point is the average of three samples consisting of five aphids each. Vertical bars are 1 s.e.

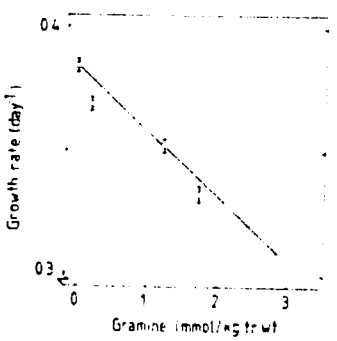

Fig. 2. Gramine content and population growth rate of *Schizaphis graminum* on barley cultivars. Nine-day-old plants were infested with two nonalate adults. Aphids were counted and leaves analysed for gramine content after 6 days. Values represent the average of three samples of five plants each. Vertical bars are 1 s.e. Aphid population growth rate = $\ln(N_t/N_0)/\Delta t$.

Effects of gramine on aphids

Gramine decreased survival of aphids reared on artificial diets with an ED_{50} of 0.8 mM (Fig. 3) after 24 hr of feeding. In addition, gramine also decreased diet intake by aphids with an ED_{50} of 2.1 mM after 5 hr of feeding.

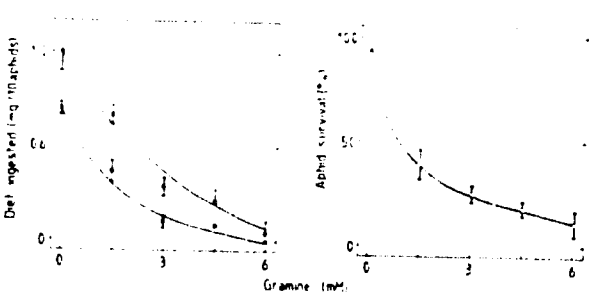

Fig. 3. Effect of gramine on diet ingestion and survival of nymphs of *Schizaphis graminum*. Diet ingestion was the difference in wt of the diet between the beginning of the experiment and at 5 (●) and 7 (○) hr. Survival was measured after 24 hr of feeding. Each point is the mean of three samples consisting of 10 aphids each. Vertical bars are 1 s.e.

Lower gramine concentrations decreased reproduction (Fig. 4). Thus, gramine had deleterious effects on aphids at concentrations similar to those found in barley leaves.

DISCUSSION

Since indole alkaloids cause acute toxicity and decrease palatibility of several grasses to mammals, it has been proposed that alkaloid content of various Gramineae be reduced by plant breeding [9, 17]. Also, it has been suggested that gramine accumulation in barley plants grown in hot environments may have potentially deleterious consequences, both for the productivity of livestock and for the heat tolerance of the crop itself [9].

This and previous work [16] have shown that gramine causes toxicity, feeding deterrence and decrease in reproduction rate of aphids reared in artificial diets. Also, gramine content correlated with resistance to the greenbug *S. graminum*. Moreover, gramine concentration in barley is sufficiently high to cause toxicity or feeding deterrence to aphids. Thus, it is likely that gramine plays a role in protecting barley from aphid attack.

Eliminating or decreasing gramine content of plants by plant breeding may be convenient for feeding livestock but it may also increase susceptibility of the crop to insects and other herbivores. It would be advisable to study alternative resistance mechanisms of this plant to insects before recommending the selection of cultivars with a low gramine content.

EXPERIMENTAL

Aphids. Individuals of the greenbug *Schizaphis graminum* (Rondani) were reared on *Hordeum distichum* cv. F. Union. Plants and aphids were kept at 28° under permanent light.

The diet was a pH 6 soln of 35% sucrose, amino acids and mineral salts [16], placed between two layers of Parafilm M [18]. All feeding experiments were performed at 28° under permanent fluorescent light.

Infestation experiments. Seed were planted in pots filled with soil and grown under continuous light in a greenhouse (23–28°). Plants were infested with nonalate adults and covered with a nylon net.

Preparation of plant extracts for benzyl alcohol analysis. Barley leaves (30–50 g) of various ages grown in the dark or under light were macerated with a mortar and pestle. The extract was filtered through cheesecloth, pH adjusted to 3 with 1 M HCl and

centrifuged to 5000 *g* for 5 min. The aq. extract was divided into 4-ml aliquots to which benzyl alcohol was added to reach various concns (0, 0.2, 0.4, 0.6, 0.8 and 1.0 mM). These solns were extracted into EtOAc. Benzyl alcohol recovery was 88%. These samples were used for analyses in a Perkin-Elmer 900 gas chromatograph equipped with FID and a 3% Carbowax 20 M glass column (2 m × 3 mm). Nitrogen (40 ml/min) was used as a carrier. Chromatograms were performed under isothermal conditions (135°). Benzyl alcohol and int. standard (3-phenyl-1-propanol) had retention times of 3.3 and 6.5 min, respectively.

Preparation of extracts for gramine analysis. Plant leaves (2–3 g) were frozen and then macerated using a mortar and pestle with 20 ml MeOH–NH_4OH (100:1). The extract was filtered through glass-wool. The filtrate was evapd and the residue was dissolved in 5 ml 0.1 M HCl. This soln was filtered in Whatman No 1 paper, adjusted to pH 9 with conc NH_4OH and extracted into $CHCl_3$ (× 2). Gramine recovery under these conditions was 97%. The organic phase was evapd to dryness. These extracts were used for gramine analyses. Indoles form colored complexes with Ehmann's reagent [19]. Gramine forms a pink complex (λ_{max} 550 nm, ε 4207). Each extract was also analysed by TLC (silica gel GF 254 plate, MeOH–NH_4OH (7:1), gramine R_f 0.55). Gramine was the only indole alkaloid in barley detected by this procedure.

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DECOMPOSITION IN APROTIC SOLVENTS OF 2,4-DIHYDROXY-7-METHOXY-1,4-BENZOXAZIN-3-ONE, A HYDROXAMIC ACID FROM CEREALS

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(*Received in UK 4 October 1984*)

Abstract – The decomposition of the title compound (DIMBOA, **1**) in aprotic solvents was analysed in terms of linear solvation energy relationships using donor numbers. The results indicate rate-limiting cyclic hemiacetal opening in low donor number solvents and rate-limiting isocyanate formation in high donor number solvents. The addition of H_2O to DIMBOA decomposing in high donor number solvents had no effect upon the reaction rate, allowing one of the two proposed mechanisms to be rejected.

Extracts of certain Gramineae such as rye, wheat and maize contain hydroxamic acids[1] which inhibit growth and development of plant pathogens[2,3] and are involved in cereal resistance to various insects.[4,5] Knowledge of the reactivity of these compounds in solution is essential for the molecular interpretation of their widespread toxicity.[6,7]

Two mechanisms for the decomposition of these acids in aqueous solutions have been proposed. Both coincide in proposing the fast opening of the hemiacetal as the first step. In one mechanism,[8] an isocyanate intermediate (**3**) would be formed in the rate-limiting step of the reaction by attack of the hydroxamic oxygen atom on the aldehyde function of the α-ketoaldehyde (**2**) (Scheme 1, path A). In the other,[9] cyclisation of **2** would form the 5-membered hemiketal **5**, water would then add in the rate-limiting step to the aldehyde function of **5** converting it to a better electrophilic leaving group and finally formic acid and water would be formed from this electrophilic residue and the hydroxamic hydroxyl group, leaving a compound which tautomerizes to the corresponding benzoxazolinone (**4**). The participation of water may be assessed by studying the effect of added water on the decomposition reaction in aprotic solvents. In this paper, we describe the decomposition of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA, **1**), the main hydroxamic acid in maize extracts,[10] in aprotic solvents. A preliminary account of this work has been published.[11]

EXPERIMENTAL

Isolation of DIMBOA. DIMBOA was isolated from ethereal extracts of 7-day old seedlings of *Zea mays* L. cv LH Rinconada grown under continuous light in a greenhouse at 28 ± 3°.[5] Identification was made by comparison of UV, IR and NMR spectra with reported spectra.[12–14]

Solvents. Solvents were purified and dried by described methods.[15] In addition, they were left 5 hr over 0.3 nm molecular sieves, passed through a 0.4 m column packed with molecular sieves and finally distilled under inert atmosphere.

Kinetics. Decompositions of DIMBOA (0.06 M) were carried out in vessels fitted with teflon-coated silicon septa. Kinetics were followed by treating aliquots of the reaction mixture with $FeCl_3$ reagent (50 g $FeCl_3 \cdot 6\,H_2O$, 500 ml 95% EtOH and 5 ml 10 N HCl) and measuring the absorbance at 590 nm. Absorbances of blanks made with aliquots treated with the solvent of the $FeCl_3$ reagent were subtracted from the absorbance of each sample. Reactions followed first order kinetics for the least 3 half-lives. Standard errors of the rate constants were lower than 4%. Activation parameters were determined from measurements at 3 or 4 different temperatures.

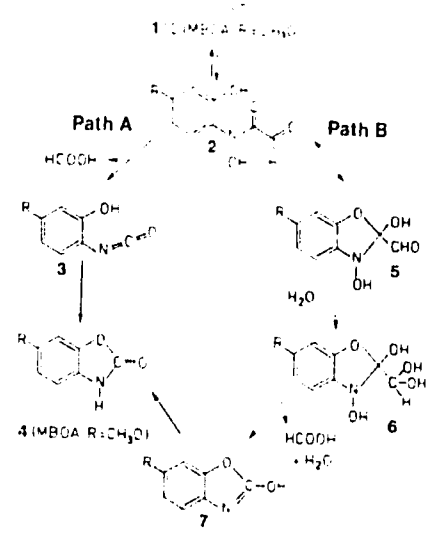
Solubility of DIMBOA. Solns of DIMBOA in equilibrium with solid DIMBOA were prepared in various solvents at 11°. Aliquots were filtered at different time intervals and analysed with $FeCl_3$ reagent. Saturation was assumed when the concentration of DIMBOA in the filtrate did not change with time. Experiments were carried out in triplicate. Standard deviations from the mean were less than 10%.

IR spectra. These were recorded in a Perkin–Elmer 621 spectrophotometer with 0.5 mm light path NaCl cells.

Products studies. The main decomposition product of DIMBOA was 6-methoxy-benzoxazolin-2-one (MBOA, **4**), as judged by its melting point (152–153°) and comparison of UV, IR and NMR spectra with reported spectra.[16]

RESULTS

The main decomposition product of 1,4-benzoxazin-3-ones was the corresponding benzoxazolinone both in



Scheme 1.

* Author to whom correspondence should be addressed.

aqueous[16] and non-aqueous solutions,[11] suggesting that the decomposition mechanisms prevailing in both types of solvents are similar.

Although it is reasonable to accept that the opening of the hemiacetal (**1** ⇌ **2**) in aqueous media is fast compared with subsequent steps in the decomposition reaction, the situation might differ in aprotic solvents. Evidence of a change in the rate-limiting step of a reaction provoked by a change in reaction medium may be obtained through a study of changes in activation parameters with medium.[17]

Rate studies

Rate constants are collected in Table 1. Solvent effects on $\Delta G^{\ddagger}$ were comparatively small. Hence, values of $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ were obtained. They were examined using donor numbers of the solvents, a measure of their ability to donate an electron pair.[18] Two different ranges of $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ were obtained: one for low donor number solvents ($\Delta S^{\ddagger} = -250$ to -170 J mol^{-1} K^{-1}; $\Delta H^{\ddagger} = 33$ to 59 kJ mol^{-1}) and another for high donor number solvents ($\Delta S^{\ddagger} = -170$ to 13 J mol^{-1} K^{-1}; $\Delta H^{\ddagger} = 50$ to 120 kJ mol^{-1}). Within each range, activation parameters correlated linearly with donor number (Fig. 1). An isokinetic relationship was obtained (Fig. 2) with a value of β (298 K) falling within the range expected for solvent effects on reaction rates and equilibria.[17] The proximity of the experimental temperature to the isokinetic temperature presumably accounts for the insensitivity of $\Delta G^{\ddagger}$ to solvent.

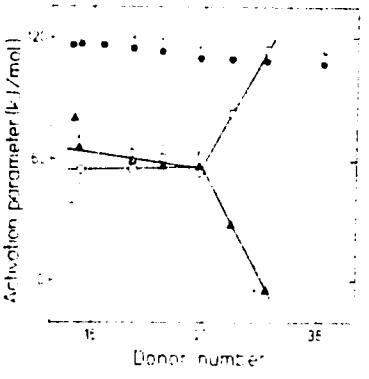


Fig. 1. Activation parameters at 326 K for the decomposition of DIMBOA in aprotic solvents: ●($\Delta G^{\ddagger}$); ○($\Delta H^{\ddagger}$); ▲($-T\Delta S^{\ddagger}$). Acetonitrile (1), dioxane (2), acetone (3), tetrahydrofuran (4), trimethylphosphate (5), N,N-dimethylformamide (6), dimethylsulfoxide (7), pyridine (8), hexamethylphosphoramide (9).

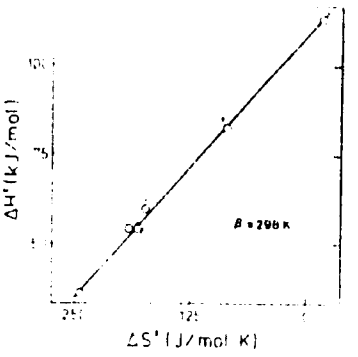


Fig. 2. Isokinetic relationship for the decomposition of DIMBOA in aprotic solvents. Acetonitrile (1), dioxane (2), tetrahydrofuran (3), trimethylphosphate (4), N,N-dimethylformamide (5), dimethylsulfoxide (6), pyridine (7). Correlation coefficient: 0.996.

Water was added to dry high donor number solvents until concentrations ranging from 0.01 to 0.4 M were obtained. This range covered the initial concentration of DIMBOA (0.06 M). The decomposition rates varied erratically within the limits of confidence of the measurements (Table 2).

Insight into the molecular interpretation of activation parameters was obtained through a study of the interaction of DIMBOA with solvent.

Interaction of DIMBOA with solvents

These interactions were assessed by measuring the solubilities and IR spectra of DIMBOA in various solvents.

The logarithms of solubilities correlated linearly with the donor number of the solvents (Fig. 3), pointing to the interaction of the solvent with electrophilic sites in DIMBOA. The nature of this interaction was probed by IR studies of the OH stretching region of solutions of

Table 2. Effect of addition of H_2O on the decomposition rates of 0.06 M DIMBOA in aprotic solvents at 53

Solvent	H_2O added/M	$10^4 k_{obs}$/min^{-1}
Dimethylsulfoxide	0.01–0.8	1.08 ± 0.21[a]
Dimethylformamide	0.09–0.34	0.83 ± 0.05[a]
Pyridine	0.03–0.36	1.43 ± 0.11[a]

[a] Average of 5, 5 and 4 points in dimethylsulfoxide, dimethylformamide and pyridine respectively.

Table 1. Rates of decomposition of 0.06 M DIMBOA at different temperatures in various solvents

Solvent	Rate constants 10^4 min^{-1} (temperature/K) $k_1(T_1)$	$k_2(T_2)$	$k_3(T_3)$	$k_4(T_4)$
Acetonitrile	1.29 (326)	1.76 (326)	2.90 (346)	
Dioxane	1.10 (326)	1.85 (336)	3.36 (346)	
Dioxane + 0.23 M H_2O	1.61 (333)		7.25 (345)	21.7 (353)
Acetone	1.17 (326)			
Tetrahydrofuran	1.25 (316)	2.38 (326)	4.52 (336)	
Dimethylformamide	8.3 (326)	19.1 (336)	27.3 (346)	
Dimethylsulfoxide	10.8 (326)	26.2 (336)	54.5 (346)	163.0 (356)
Dimethylsulfoxide + 0.23 M H_2O		22.6 (336)	61.3 (346)	150.0 (356)
Pyridine	14.3 (326)	43.3 (336)	152.0 (346)	
Hexamethylphosphoramide	26.0 (326)			

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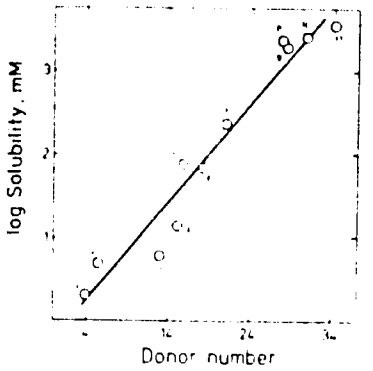

Fig. 3. Solubilities of DIMBOA in aprotic solvents. Nitromethane (1), nitrobenzene (2), benzonitrile (3), acetonitrile (4), dioxane (5), acetone (6), tetrahydrofuran (7), N,N-dimethylformamide (8), N-methyl-2-pyrrolidone (9), dimethylsulfoxide (10), pyridine (11). Correlation coefficient: 0.945.

DIMBOA. The spectra showed absorptions corresponding to unassociated (3700–3400 cm^{-1}) and to associated (3300–3200 cm^{-1}) OH groups. At concentrations lower than 1 mM, hydroxyl groups of DIMBOA were essentially unassociated. Two bands were present in the free OH region of the spectra corresponding to the two hydroxyl groups of DIMBOA. As the donor number of the solvent increased the bands approached one another and were displaced towards the associated OH region. The absorption frequencies correlated with solvent donor number (Fig. 4), reflecting hydrogen bonding to solvent with the corresponding lengthening and weakening of the OH bond.[18,19]

DISCUSSION

The opening of the hemiacetal in DIMBOA requires the transfer of a proton from the hydroxyl group in C_2 to the ring oxygen atom. The rate of this transfer is related in aprotic solvents to the interaction of the proton with solvent through nucleophilic sites in the latter (Fig. 4). It is conceivable that depending on the strength of these interactions, this process may be rate-limiting.

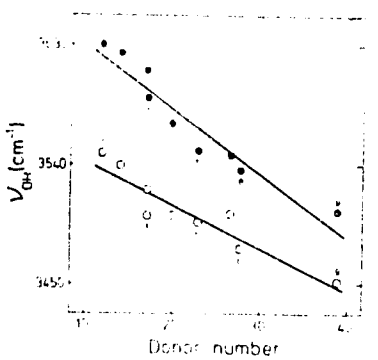

Fig. 4. Hydroxyl stretching frequencies of DIMBOA in aprotic solvents. Benzonitrile (1), acetonitrile (2), dioxane (3), ethylacetate (4), tetrahydrofuran (5), trimethylphosphate (6), N,N-dimethylformamide (7), N,N-dimethylacetamide (8), hexamethylphosphoramide (9). Correlation coefficients: ●, 0.953; ○, 0.911.

The inflection in the activation parameters (Fig. 1) may be attributed to a change in the rate-limiting step of the reaction. The range of $\Delta S^{\neq}$ in low donor number solvents points towards important differences in organization between the basal and transition states in the neighbourhood of the reaction centre. Highly negative $\Delta S^{\neq}$ values may be associated to the rate-limiting opening of the cyclic hemiacetal since a high degree of solvation of the transition state would be required for proton transfer from the hydroxyl group in C_2 to the ring oxygen atom. This difference of solvent organization would be particularly drastic in low donor number aprotic solvents.

In support of this proposition, $\Delta S^{\neq}$ and $\Delta H^{\neq}$ values for the decomposition of DIMBOA in dry dioxane, a low donor number solvent ($\Delta H^{\neq}$ = 54.3 kJ mol^{-1}; $\Delta S^{\neq}$ = −191 J mol^{-1} K^{-1}), were substantially different from those values in dioxane with H_2O added ($\Delta H^{\neq}$ = 116 kJ mol^{-1}; $\Delta S^{\neq}$ = 7.5 J mol^{-1} K^{-1}), in spite of the similarity between free energies of activation ($\Delta G^{\neq}$ = 117 kJ mol^{-1} in dry dioxane and 114 kJ mol^{-1} in aqueous dioxane). In the first case, hemiacetal opening would be rate-limiting and the activation parameters fall on the line for other low donor number solvents. In the presence of H_2O, hemiacetal opening would become fast and the activation parameters now fall in the range for high donor number aprotic solvents. In contrast, activation parameters in dry dimethylsulfoxide, a high donor number solvent ($\Delta H^{\neq}$ = 88 kJ mol^{-1}; $\Delta S^{\neq}$ = −69 J mol^{-1} K^{-1}), were not substantially different from those in dimethylsulfoxide with H_2O added ($\Delta H^{\neq}$ = 91 kJ mol^{-1}; $\Delta S^{\neq}$ = −60 J mol^{-1} K^{-1}). The influence of added H_2O was negligible ($\Delta G^{\neq}$ = 110 kJ mol^{-1} in dry dimethylsulfoxide, $\Delta G^{\neq}$ = 111 kJ mol^{-1} with H_2O added).

Additionally, activation parameters in acetonitrile deviated from the linear correlation (Fig. 1). The extra negative contribution to $T\Delta S^{\neq}$ in acetonitrile may be the reflection of the participation of this solvent not only as nucleophile but also as electrophile due to the higher electrophilic character of this solvent relative to the others studied. Indeed, the acceptor donor number ratio is approximately double for acetonitrile (1.34) than for other low donor number solvents employed (dioxane = 0.73, tetrahydrofuran = 0.44, dimethylformamide 0.6).

Hence, the possibility of distinguishing the proposed decomposition mechanisms of DIMBOA based on the addition of H_2O is limited to those solvents where hemiacetal opening is fast, i.e. high donor number aprotic solvents, and those where additional solute–solvent interaction mechanisms are absent. Table 2 shows that added H_2O has no significant effect on the decomposition rate of DIMBOA in high donor number aprotic solvents, suggesting that path B (Scheme 1) is not the predominant mechanism for the decomposition of DIMBOA.

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THE REDUCTION OF 2,4-DIHYDROXY-7-METHOXY-1,4-BENZOXAZIN-3-ONE BY THIOLS

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(*Received* 13 *May* 1985)

Key Word Index—*Zea mays*; Gramineae; 1,4-benzoxazin-3-one; thiols; reduction.

Abstract—2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), a naturally occurring hydroxamic acid involved in pest resistance of cereals, was reduced by thiols to the corresponding lactam. Kinetic studies showed that the reactive species are undissociated DIMBOA and thiolate anion. Possible mechanisms for the reaction are discussed in the light of relative reactivities of DIMBOA and a compound lacking the 7-methoxy substituent, and results from molecular orbital calculations.

INTRODUCTION

Hydroxamic acids isolated from extracts of Gramineae such as wheat, maize and rye [1] inhibit bacterial [2] and fungal [3] growth, as well as insect development and reproduction [4, 5]. These compounds have been suggested as resistance factors of maize against the European corn borer [4], and of cereals against aphids [6]. The main hydroxamic acid in maize is 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA, **1**) [7]. It was recently shown that DIMBOA inhibits energy transfer reactions in chloroplasts and mitochondria [8, 9], a fact that may account for its widespread toxicity [8]. These enzymatic inhibitions were shown to be partly due to the reaction of DIMBOA with sulfhydryl groups of the enzymes [9]. To gain further insight into these biochemical reactions we undertook the study of the reaction of DIMBOA with thiols. A preliminary account of this study has been published [10].

RESULTS

Spectroscopic description of the reaction

The reaction of DIMBOA with thiols was followed between pH 5 and 12 through the spectral changes in the 240–310 nm region. In this pH range the main product was the corresponding lactam, **5**.

At pH about 8, the starting spectrum was that of dissociated DIMBOA with λ_{max} at 290 nm. As the reaction proceeded this absorption peak gradually decreased, a peak at 259 nm corresponding to the lactam gradually increased and an isosbestic point at 271 nm was produced. At pHs below 6, the starting spectrum was that of undissociated DIMBOA with λ_{max} at 263 and 286 nm, the absorption of the product at 259 nm thus being partially hidden by the reactant. At pHs above 9, thiolate anion absorbs strongly below 280 nm [11] and the absorption of the product remained hidden. Acidification of this solution to pH 8 revealed the absorption at 259 nm due to the product. The reaction was hence followed in all cases at 290 nm, where the lactam shows negligible absorption. Additionally, at pH around 8 the reaction could equivalently be monitored at 259 nm.

Kinetics

DIMBOA disappeared in the presence of an excess of thiol with pseudo-first order kinetics. The rate law for the reaction is given by equation (1). DH_2 represents

$$-d(DH_2)_t/dt = k_{obs}(DH_2)_t = k_1(DH_2)_t + \bar{k}_2(DH_2)_t(RSH)_t \quad (1)$$

DIMBOA as a diprotic acid and RSH represents a thiol. Subscripts 't' indicate total concentrations (formalities); k_1s are first order rate constants for the pH-dependent decomposition of DIMBOA [12]; $\bar{k}_2$s are apparent second order rate constants for the reaction of DIMBOA with thiols, and were determined from the slope of plots k_{obs} vs. thiol concentration. Figure 1 shows such plots at different pHs for the reaction of DIMBOA with mercaptoethanol. Figure 2 shows the dependence of $\bar{k}_2$ on pH at a given concentration of thiol. The curves are bell-shaped with maxima at pH 8.3 for the reaction with mercaptoethanol, and 6.7 for the reaction with cysteine methyl ester.

The distribution of species present in a solution of DIMBOA and mercaptoethanol or cysteine methyl ester, were calculated using pK_a values from the literature [12, 13]. The products $(DH_2)(RS^-)$ and $(DH^-)(RSH)$ varied with pH in the same way as the experimental curves of Fig. 2. The following identities can thus be established, where the subscripts 'e' indicate effective concentrations, k_2 true pH-independent second order rate constants for the reaction, and 'f' molar fractions.

$$\bar{k}_2(DH_2)_t(RSH)_t = k_2(DH_2)_e(RS^-)_e \text{ or } k_2(DH^-)_e(RSH)_e \quad (2)$$

$$k_2 = \bar{k}_2/f_{DH_2}f_{RS^-} \text{ or } \bar{k}_2/f_{DH^-}f_{RSH} \quad (3)$$

*Author to whom correspondence should be addressed.

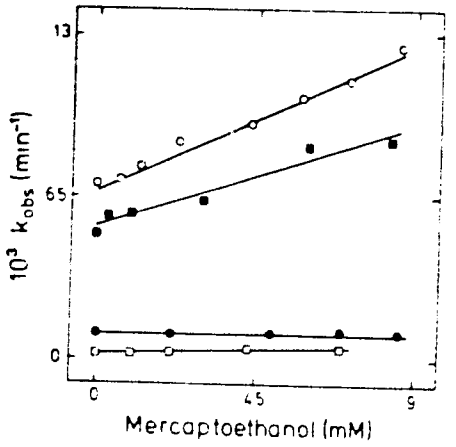


Fig. 1. Variation of the observed pseudo-first order rate constants for the reaction of DIMBOA with mercaptoethanol, with thiol concentration at pH 5.0 (□), 12.1 (●), 7.2 (■) and 8.1 (○).

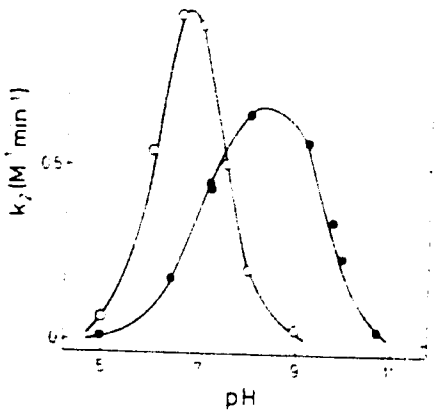


Fig. 2. pH dependence of the apparent second order rate constant $\bar{k}_2$ for the reaction of DIMBOA with mercaptoethanol (●) or with cysteine methyl ester (○). Values of $\bar{k}_2$ were determined from the slopes of graphs of k_{obs} against thiol concentration (see Fig. 1).

In order to distinguish between the two situations presented, k_2 values for a series of thiols were determined. For mercaptoethanol and cysteine methyl ester, k_2s were obtained from the least-squares fit shown in Fig. 2. For cysteine, dithiothreitol, thiolactic acid and mercaptoacetic acid, k_2s were determined from one point experiments using equation 3 for DIMBOA and thiolate anion. A linear correlation was obtained when log k_2 was plotted against the corresponding pK_a of the thiol [11, 13, 14] (Fig. 3), indicating that the reactive species were undissociated DIMBOA and thiolate anion.

Comparative reactivity studies

The reactivity towards thiols of DIMBOA was compared with that of DIBOA (a naturally occurring analogue of DIMBOA lacking the methoxy group). pK_a values of both hydroxamic acids were determined under the same experimental conditions. At 44°, the pK_a of DIMBOA was 6.74 ± 0.05 and that of DIBOA 6.79 ± 0.04.

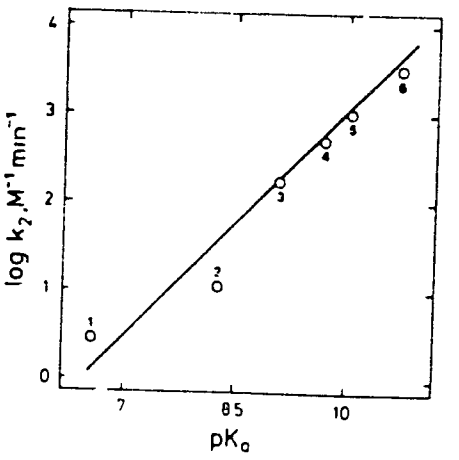


Fig. 3. Dependence of the logarithm of the true second order rate constant for the reaction of DIMBOA with thiols on the pK_a of the thiol. The true second order rate constant k_2 for each thiol was determined by least-squares fitting of $\bar{k}_2$ to equation

$$k_2 = \bar{k}_2\left[\frac{(H^+)^2 + K_1(H^+) + K_1K_2}{(H^+)^2}\right]\cdot\left[\frac{(H^+) + K_3}{K_3}\right]$$

where $K_1 = 1.25 \times 10^{-7}$ and $K_2 = 1.26 \times 10^{-11}$ are the dissociation constants of DIMBOA [12], and K_3 is the dissociation constant of the thiol. Thiols used were: cysteine methyl ester (1), cysteine (2), dithiothreitol (3), mercaptoethanol (4), thiolactic acid (5) and mercaptoacetic acid (6).

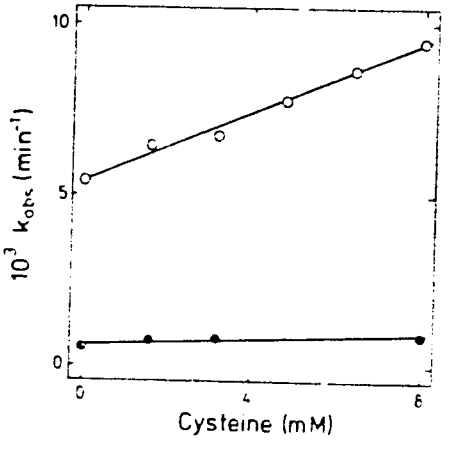


Fig. 4. Variation of the observed pseudo-first order rate constant for the reaction of DIMBOA (○) and DIBOA (●) with cysteine at pH 7.5 (phosphate 0.1 M) and 44 ± 0.5°.

Hence, it can be assumed that relative reactivity of both hydroxamic acids towards thiols (relative k_2) are equivalent to relative $\bar{k}_2$ at any given pH. A plot of k_{obs} vs. cysteine concentration (Fig. 4) shows the substantial influence of the 7-methoxy group upon reactivity towards thiols.

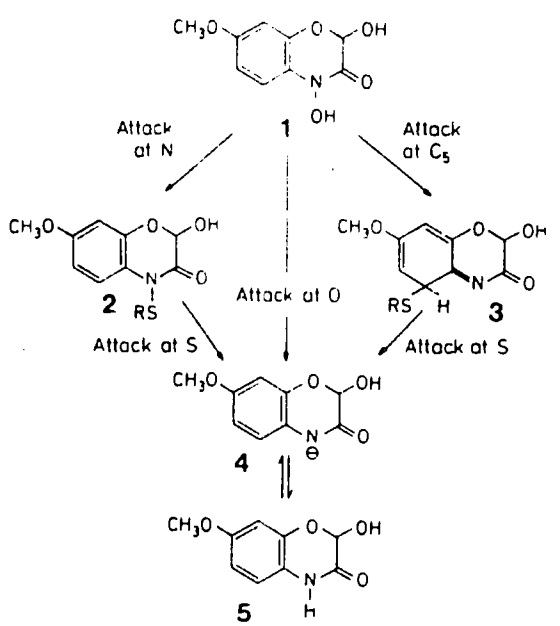
DISCUSSION

Recently it was reported that DIMBOA inhibits ATPase activity of chloroplast coupling factor (CF_1) and it was suggested that part of this inhibition was caused by

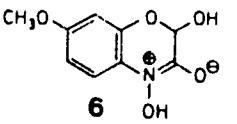
the reaction of DIMBOA with sulfhydryl groups on the enzyme. Sulfhydryl groups in proteins may vary widely in reactivity due to factors such as intrinsic nucleophilicity, local chemical microenvironment and steric hindrance [11, 15]. We undertook the study of the influence of these factors on the reaction of DIMBOA with thiols in aqueous solutions. In a previous communication [10] we reported that the main product of the reaction was the lactam of DIMBOA, 5. The present work describes the dependence of the reactivity of DIMBOA towards thiols on pH, on the nucleophilicity of the thiol and on the effect of the 7-methoxy substituent. This work also shows that undissociated DIMBOA and thiolate anion are the most reactive couple.

The absence of ESR signals during the reaction suggests that the reaction is unlikely to proceed through a radical mechanism. Several ionic mechanisms can be envisaged that account for the results presented. DIMBOA has at least three electrophilic centres susceptible to attack by thiols to give its lactam (Scheme 1). It should be pointed out that all the species written may exist in aqueous solution in open hemiacetal form [10, 12, 16–18]. CNDO/2 molecular orbital calculations were performed to assess the relative reactivity of these centres. Nucleophilic superdelocalizability [19], a measure of the tendency of an atom to interact with a nucleophile, was higher for the hydroxamic nitrogen atom, indicating it as the most likely candidate for attack by thiolate anion. Attack at electrophilic nitrogen by thiols is not without precedent in the literature [20–22]. Intermediates similar to 2 have been isolated from the reaction of 7-methoxy-4-acetoxy-1,4-benzoxazin-3-one with ethanethiol [22].

The substantial rate increase caused by the 7-methoxy group in DIMBOA may be rationalized in terms of resonance structure 6. This is borne out by molecular orbital calculations which show a higher polarization of the carbonyl bond and a lower electron density at the hydroxamic nitrogen atom in DIMBOA as compared with DIBOA. Similar situations have been reported where introduction of a 7-methoxy group in 1,4-benzoxazin-3-



Scheme 1.



ones enhances the reactivity of the hydroxamic nitrogen atom towards nucleophiles [22].

EXPERIMENTAL

Isolation of compounds. DIMBOA was isolated as described [2] from Et_2O extracts of 6-day-old seedlings of *Zea mays* L. cv T129s grown in a greenhouse at 25 ± 3°. Mercaptoethanol, dithiothreitol, thiolactic acid and mercaptoacetic acid (Sigma) were used without further purification. Cysteine methyl ester was obtained as described [13].

The reduction product of DIMBOA was isolated from Et_2O-extracts of the reaction of DIMBOA with dithiothreitol in KH_2PO_4 (0.1 M, pH 8) and was characterized as the corresponding lactam 5 by comparison of its UV, IR (KBr) and mass spectra with those reported previously [10].

Synthesis of 2-bromo-4-hydroxy-1,4-benzoxazin-3-one. A mixture of 1 g (0.006 moles) of 3,4-dihydro-4-hydroxy-1,4-benzoxazin-3-one (previously synthesized as described [23]) and 1.2 ml (0.012 moles) trichlorobromomethane in 10 ml CCl_4 was refluxed and stirred overnight. The reaction mixture was cooled to room temp., filtered and the filtrate concd to dryness. The residue was recrystallized from petrol to give (25% yield) the desired compound. The 60 MHz NMR spectrum (CCl_4, TMS) of the bromo compound was similar to that of the starting material except for the signal of the proton at C-2: in the initial compound it appeared at δ4.6 (s, 2H) whereas in the product it was shifted to δ4.8 (s, 1H).

Synthesis of 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA). A mixture of 100 mg (0.55 mmoles) 2-bromo-4-hydroxy-1,4-benzoxazin-3-one and 42 mg (0.5 mmole) $AgCO_3$ in 20 ml wet Et_2O was stirred for 3 hr at room temp., acidified to pH 5, filtered and the filtrate concd to dryness. The residue was recrystallized from petrol to yield DIBOA, which was characterized by comparison of its mp, R_f in TLC and UV spectrum with the lit. [24].

Kinetic measurements. To 10 ml of 0.1 M buffer soln of the desired pH, thiol was added to obtain an 8 mM reference soln. Five ml of this soln were pipetted into a tube containing enough solid DIMBOA to obtain a 0.08 mM soln (sample soln). The reactions were monitored in the 240–310 nm region using quartz cells thermostatted at 31 ± 0.2°. pH was measured before and after each reaction.

pK_a measurements. The pK_a values of DIMBOA and DIBOA were determined spectrophotometrically at 44 ± 0.5° using buffers with $I = 0.1$. Spectra were recorded in the region 220–350 nm for each pH value. Isosbestic points were obtained up to pH 9 (at 272.5 nm for DIMBOA and 267.5 nm for DIBOA) indicating the presence in equilibrium of two absorbing species [25]. The analytical wavelengths employed were 260 nm for DIMBOA and 250 nm for DIBOA.

Electron spin resonance studies. ESR spectra were recorded with a Varian V4502 spectrometer. Measurements were performed at room temp. with $H_0 = 3340$ Gauss and $\nu = 9.5 \times 10^9$ Hertz. Under these conditions no signal was detected from a mixture of 0.02 M DIMBOA and 1 M mercaptoethanol in borate buffer pH 8 even after 10 min of reaction time.

Molecular orbital calculations. The CNDO/2 method [26, 27] was used with the geometry of one of the two independent molecules in the asymmetric unit of crystalline DIBOA [28]. The

methoxy group of DIMBOA was described with average crystallographic parameters [29], the rest of the molecule was identical to DIBOA.

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EFFECT OF BORATE ON THE DECOMPOSITION OF 2,4-DIHYDROXY-7-METHOXY-1,4-BENZOXAZIN-3-ONE, A HYDROXAMIC ACID FROM GRAMINEAE

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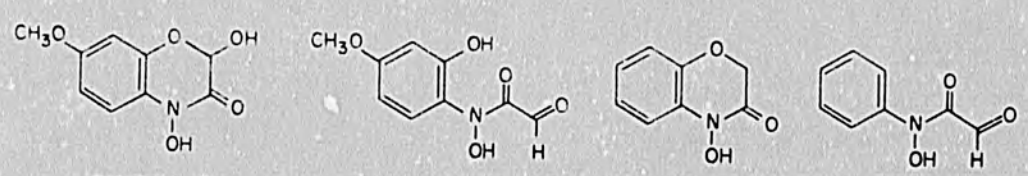
Abstract — Borate anion catalyzed the decomposition of the title compound, a resistance factor of Gramineae. The effect was attributed to the formation of a complex between borate and the open-chain hydroxamic acid intermediate in the decomposition reaction.

Cyclic hydroxamic acids isolated from extracts of Gramineae (Hx) play an important role in the defense of the plant against insects.[1,2] In addition, they exhibit toxic effects towards several bacteria[3] and fungi[4], and have been reported as mutagenic to bacteria.[5] While studying the aqueous decomposition of DIMBOA, **1**, the main Hx in wheat and maize extracts, a catalytic effect by borate buffer was observed. In this paper this effect is described and an interpretation of its origin suggested.

The decomposition of DIMBOA was monitored spectrophotometrically at 290 nm. The main decomposition product was 6-methoxy-benzoxazolin-2-one. First order rate constants were calculated using a linear-regression computer program.[6] Analogues of DIMBOA were synthesized according to reported methods.[7,8]

The catalytic effect of borate is shown in Fig. 1. Hydroxamic acids have been shown to interact with borate.[9] However, DIMBOA decomposes with the intermediacy of the open-chain hydroxamic acid **2**.[8,10,11] In order to determine which of these two hydroxamic acids interacts with borate, model compounds **3** and **4** were synthesized and their spectra recorded (Fig. 2).



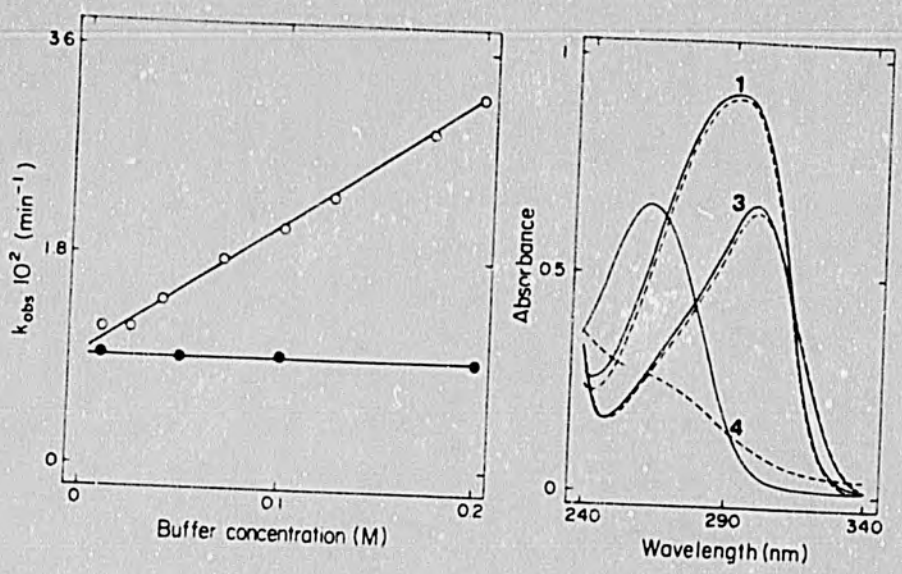
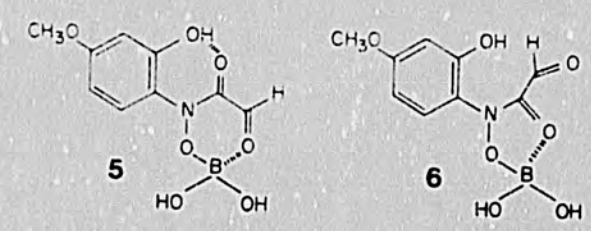


Fig. 1. Effect of borate ( ) and phosphate ( ) pH 9 on the rate of decomposition of DIMBOA (8 10^{-5} M) at 31°.

Fig. 2. Absorption spectra of 8 10^{-5} M solutions of compounds **1**, **3** and **4** in 0.1 M phosphate (---) and borate (---) pH 9 buffers.

Fig. 2 shows that while the optical density of **4** at 260 nm substantially increased in borate as compared with phosphate buffer, the absorption peaks of **1** and **3** at 290 and 300 nm respectively, were not affected. These results indicate that only compound **4** interacts with borate, and give support to the proposal of complex formation between the open-chain form of DIMBOA, **2**, and borate. This complex was not detected in the spectrum of DIMBOA presumably due to the low equilibrium concentration of **2**.[12]

The structure of the complex can not be similar to that reported for other hydroxamic acids,[9] since no enolizable hydrogen is present in **2**. However, analogous interaction of borate with the hydroxamic hydroxyl moiety leads to structures **5** and **6**. From a thermodynamic viewpoint, complex **5** would be favored



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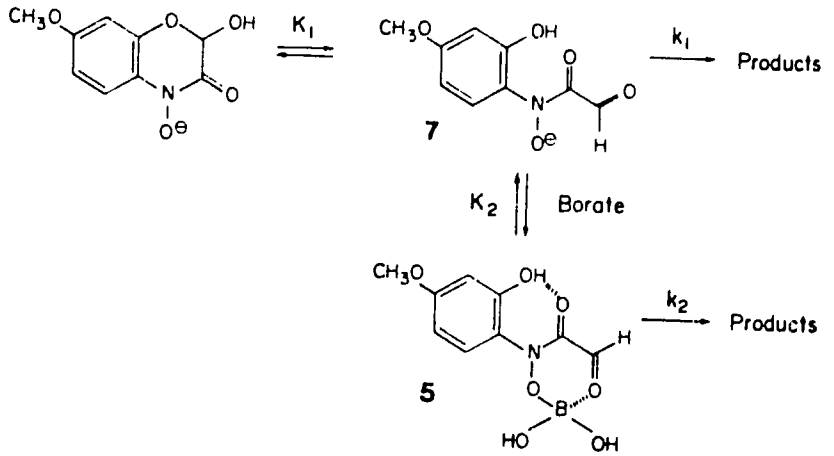
since it involves the formation of a 6-membered ring and is further stabilized by intramolecular hydrogen bonding.

The fact that cyclic hydroxamic acids 1 and 3 do not show interaction with borate may be rationalized on the basis of interatomic distances. The distance between the hydroxamic hydroxyl oxygen and the carbonyl oxygen has been determined crystallographically as 2.84 A in the analogue of DIMBOA lacking the 7-methoxy group.[13] This distance is 0.49 A longer than the O-C distance in boric acid. Thus, the formation of a complex of type 6 is less likely on this basis, and also because it would necessarily lead to a 5-membered ring.

The following scheme is hence postulated for the decomposition of DIMBOA in the presence of borate pH 9:



Under steady-state conditions, equation 1 is obtained.[14] This may be reduced to equation 2, in which k_{obs} and k^{o}_{obs} are the observed rate constants in the

$$v = \frac{K_1}{1 + K_1}(\text{DIMBOA})\left[k_1 + k_2K_2(\text{Borate})\right] \qquad (1)$$

$$\frac{v}{(\text{DIMBOA})} = k_{obs} = k^{o}_{obs} + k_{cat}(\text{Borate}) \qquad (2)$$

presence and absence of borate. This model fits the experimental results shown in Fig. 1, with a catalytic constant, k_{cat}, equal to 0.103 min^{-1} M^{-1}.

The formation of products from intermediate 2 (or 5) involve the nucleophilic attack of the hydroxamic oxygen atom on the aldehydic carbonyl group.[10,11] The catalytic effect of borate may arise from both thermodynamic and kinetic factors. Since an open-chain intermediate is necessary for reaction, the

displacement of equilibria towards this compound via formation of borate complex **5** favors the reaction. On the other hand, complex **5** is presumably more reactive than **7** on entropic grounds (reactive groups are held in suitable position for reaction) as well as on enthalpic grounds (strong polarization by boron of the aldehydic carbonyl group).

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14 In this equation, total concentration of DIMBOA has been used. DIMBOA is a diprotic acid with pK_a's of 6.9 and 10.9.[15] Since at pH 9 the mono-anion corresponds to 99.7% of total DIMBOA, the error involved in this equation is negligible.

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INHIBITION OF MITOCHONDRIAL ENERGY-LINKED REACTIONS BY 2,4-DIHYDROXY-7-METHOXY-1,4-BENZOXAZIN-3-ONE (DIMBOA), A HYDROXAMIC ACID FROM GRAMINEAE

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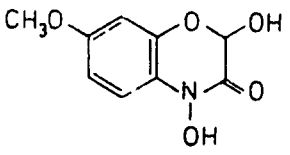
Abstract—DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) is the main hydroxamic acid isolated from maize extracts. It inhibited reversibly ATP synthesis, P_i-ATP exchange reaction and ATPase activity in submitochondrial particles from bovine heart. Half-maximal effects were obtained with 4, 2, and 6 mM DIMBOA respectively. At higher concentrations it also inhibited mitochondrial electron transport (I_{50} = 11 mM). Irreversible inactivation of mitochondrial electron transport, P_i-ATP exchange reaction and 8-anilino-1-naphthalene sulfonate energy-dependent fluorescence enhancement was also observed. These effects of DIMBOA on energy-linked mitochondrial reactions may explain the inhibitory action of DIMBOA on several aerobic organisms.

DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) is the main hydroxamic acid isolated from wheat extracts [1]. This and other hydroxamic acids, isolated from various Gramineae [2], have been suggested to play an important role in the defense of the plant against insects [3–6]. It has also been shown that they inhibit bacterial [7] and fungal [8] growth.



DIMBOA

Recently, it was reported that DIMBOA inhibits photosynthetic ATP synthesis in spinach chloroplasts, thus behaving as an energy transfer inhibitor [9]. Coupling factor CF_1 from the same origin is affected by DIMBOA in two different manners: (i) a reversible one that accounts for an uncompetitive inhibition of the ATPase activity, and (ii) an irreversible one that results in a progressive inactivation of the enzyme [10]. This last effect was suggested to be due to an irreversible modification of sulfhydryl groups in the enzyme molecule since DIMBOA is able to participate in reactions with sulfhydryl compounds forming addition and/or reduction products [11–13].

The above-described effects of DIMBOA on different organisms are indicative that hydroxamic acids can act, similarly to other products of secondary plant metabolism, as allelochemical agents, playing an important role in the interaction between different species or in the mechanism of defense against plant pathogens or predators [14, 15]. Only in a few cases is the molecular mechanism of this allelochemical action well known. One of the enzymic systems that must be considered as potential targets for the action of such allelochemical agents are the systems involved in mitochondrial energy metabolism [16, 17].

In this paper we discuss the effect of DIMBOA on electron transport and other energy-linked reactions in phosphorylating submitochondrial particles from bovine heart, as an attempt to contribute to the knowledge of the biochemical basis of its action on plant predators.

MATERIALS AND METHODS

Heavy bovine heart mitochondria were prepared as described [18]. Phosphorylating submitochondrial Mg^{2+}-ATP particles (SMP) were prepared from bovine heart mitochondria essentially as described [19].

Electron transport. Electron transport in SMP was determined following oxygen consumption with a Clark electrode connected to a Gilson oxygraph in a reaction medium containing 250 mM sucrose and 50 mM Tris–HCl (pH 7.5) using either 10 mM succinate, an NADH-regenerating system composed of 50 mM ethanol, 0.2 mM NADH and 125 I.U./ml of yeast alcohol dehydrogenase (EC 1.1.1.27) or 5 mM ascorbate *plus* 0.1 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) as oxidizable substrate. Rate values reported correspond to the slopes of linear traces drawn by the oxygraph.

Spectral studies. The redox state of the cytochromes of the respiratory chain under different conditions was studied spectrophotometrically in an Aminco DW-2a provided with a magnetic stirrer

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attachment and connected to a MIDAN-T microprocessor data analyzer.

ATPase activity. SMP (60 μg protein) were added to a reaction medium (1 ml) containing 180 mM sucrose, 2 μM carbonylcyanide *p*-trifluoro-methoxyphenyl hydrazone (FCCP) and 50 mM Tris–HCl (pH 7.5). After 5 min of preincubation, the reaction was started by adding ATP (4 mM final concn). The reaction was stopped 5 min later with 0.05 ml of 100% trichloroacetic acid (TCA). After centrifugation (10 min, 3000 rpm), aliquots were withdrawn from the supernatant fraction and analyzed for inorganic phosphate according to Sumner [20].

ATP synthesis. The reaction was measured in a medium containing (final concn) 180 mM sucrose, 1 mM $MgCl_2$, 10 mM succinate, 0.5 mM EDTA, 3 μM rotenone, 50 mM glucose, 2 mM ADP, 5 I.U. of yeast hexokinase (EC 2.7.1.1) and 50 mM Tris–HCl, pH 7.4. SMP (0.4 mg protein) were added to this medium and incubated for 5 min. ATP synthesis was started by adding 5 μmoles P_i, 2×10^6 cpm carrier free $^{32}P_i$ and 3 μmoles $MgCl_2$. The final volume was 1 ml. The preincubation and the reaction were carried out under aerobiosis obtained with a gyratory waterbath shaker. After 5 min the reaction was stopped and inorganic phosphate was quantitatively precipitated essentially as described by Sugino and Miyoshi [21]. After separating the ppt by centrifuging for 10 min at 3000 rpm, aliquots were withdrawn and analyzed for [^{32}P]glucose-6-phosphate by Cerenkov counting in a Beckman 8100 liquid scintillation counter.

P_i-ATP exchange reaction. SMP (1 mg protein) were added to 1 ml of a reaction medium containing 180 mM sucrose, 10 mM ATP, 10 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.5), 10 mM P_i and 2×10^6 cpm of carrier free $^{32}P_i$. After 5 min the reaction was stopped by adding Sugino and Miyoshi's reagent [21] and, after centrifuging at 3000 rpm for 10 min, the supernatant fraction was analyzed for [γ-^{32}P]ATP by Cerenkov counting.

Energy-linked 8-anilino-naphthalene sulphonate (ANS) fluorescence enhancement. Measurements were carried out in a Perkin–Elmer 650-40 fluorescence spectrophotometer. ANS was excited at 380 nm and fluorescence was measured at 480 nm essentially as described by Ferguson *et al.* [22].

Irreversible inactivation of SMP by DIMBOA. SMP (10 mg/ml) were incubated in a reaction medium containing 180 mM sucrose and 50 mM Tris–HCl, pH 7.5. After 5 min of preincubation, DIMBOA was added and aliquots were withdrawn at different time intervals and diluted (at least 100 times) in the appropriate reaction media in order to measure succinate oxidation, NADH oxidation, P_i-ATP exchange reaction, ATPase activity, or energy-linked ANS fluorescence enhancement.

Protein determinations were carried out using a modified biuret procedure [23].

DIMBOA was isolated as previously described [10], and dissolved in dimethyl sulfoxide (DMSO) prior to its addition to SMP. Controls with the solvent (less than 2%) were performed for all the biochemical reactions studied. Measurements were carried out at 25°. Rates are the average of determinations in duplicate which agreed within 10%.

RESULTS

DIMBOA inhibited electron transport from succinate to oxygen in submitochondrial particles (SMP) from bovine heart. The dependence of the inhibition on DIMBOA concentration followed a sigmoidal curve (Fig. 1A). Hardly any effect was observed at concentrations below 4 mM. Similar results are obtained for NADH and ascorbate + TMPD oxidations (data not shown). In all cases 50% inhibition was obtained with about 11 mM DIMBOA.

Inhibition by DIMBOA of ATP synthesis and P_i-ATP exchange reaction catalyzed by SMP was more effective and attained completeness. In both cases hyperbolic titration curves were obtained (Fig. 1B)

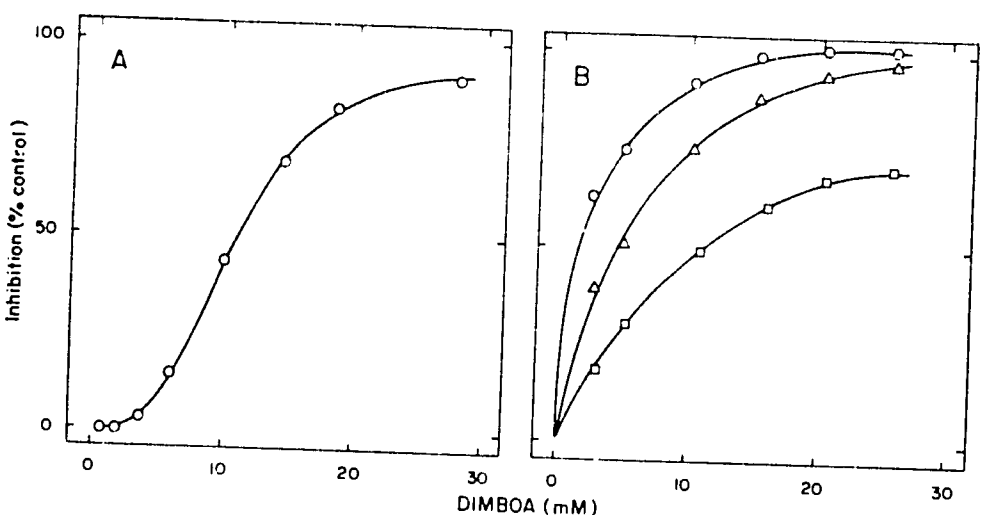

Fig. 1. Effect of DIMBOA on mitochondrial electron transport and other energy-linked reactions. (A) Mitochondrial electron transport (○) was measured as indicated in Materials and Methods using succinate as oxidizable substrate. (B) ATP synthesis (○), P_i-ATP exchange reaction (△) and ATPase activity (□) were measured as indicated under Materials and Methods. Activities for controls were 292 natoms/min · mg protein, and 315, 102, and 521 nmoles/min · mg protein respectively.

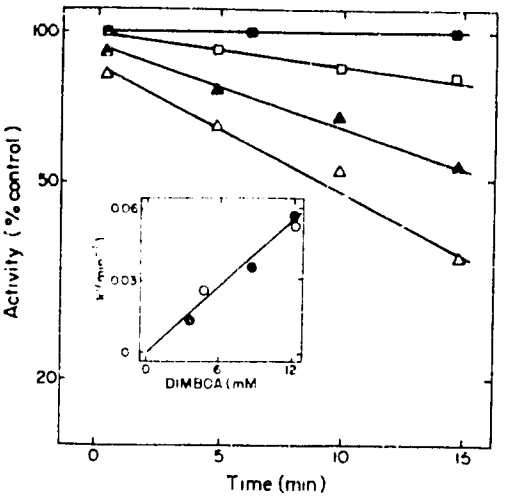

Fig. 2. Irreversible inactivation of mitochondrial electron transport by DIMBOA. SMP were incubated with 0 (■), 4 (□), 9 (▲) and 12.5 mM (△) DIMBOA as described in Materials and Methods. At the indicated time intervals, aliquots were withdrawn and diluted in the media for determining electron transport from succinate to oxygen. The pseudo-first-order rate constants (k') calculated from the slopes of the semilogarithmic plots are shown in the inset (●). Open circles (○) correspond to similarly determined values obtained from inactivation plots of electron transport from NADH to oxygen. Activities for controls were 278 (succinate) and 161 (NADH) natoms/min·mg protein.

that allowed the determination of I_{50} values (4 and 2 mM DIMBOA respectively) from double-reciprocal plots of percent inhibition versus DIMBOA concentration. DIMBOA also inhibited ATPase activity but the maximal inhibition that could be obtained

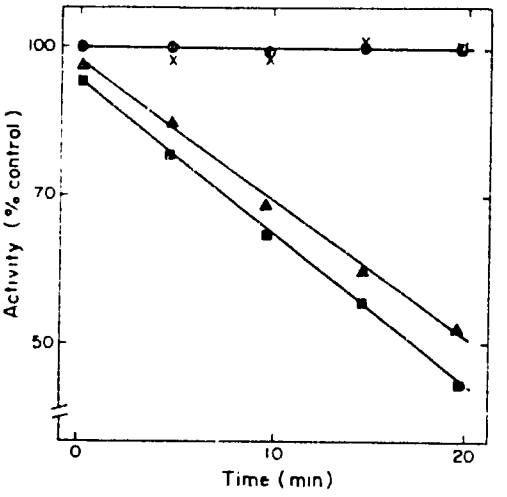

Fig. 3. Protection by succinate of mitochondrial electron transport against inactivation by DIMBOA. SMP were treated with 15 mM DIMBOA in the absence (■ and ▲) or in the presence of 10 mM succinate (● and ×) and then assayed for succinate oxidation (▲ and ●) or NADH oxidation (■ and ×) essentially as described in Materials and Methods. When NADH oxidation was measured, 1 mM malonate was added in order to specifically inhibit succinate oxidation. Activities for controls were 284 (succinate) and 172 (NADH) natoms/min·mg protein.

was about 70% (Fig. 1B). Half-maximal effect was exerted by 6 mM DIMBOA.

The above-mentioned effects were produced almost instantaneously. When succinate oxidation was measured after incubating SMP with DIMBOA, a progressive inactivation was observed (Fig. 2). This must be attributed to an irreversible reaction because it persisted after 100-fold dilution and the activity of diluted samples did not change after standing for 30 min. This inactivation followed pseudo-first-order kinetics with a rate constant (k') that depended linearly on DIMBOA concentration (Fig. 2, inset). Similar results were obtained when NADH oxidation was measured with DIMBOA-treated SMP (see open circles in Fig. 2, inset). The inactivation of succinate and NADH oxidations by DIMBOA was protected completely by 10 mM succinate (Fig. 3). Spectral studies showed that the reduction of cytochromes *b*, *c* and *a* by succinate was blocked almost completely by preincubation of SMP with DIMBOA (Fig. 4, spectra B and D). However, under the same conditions, cytochromes *c* and *a* were reduced by ascorbate + TMPD (Fig. 4, spectra F and C).

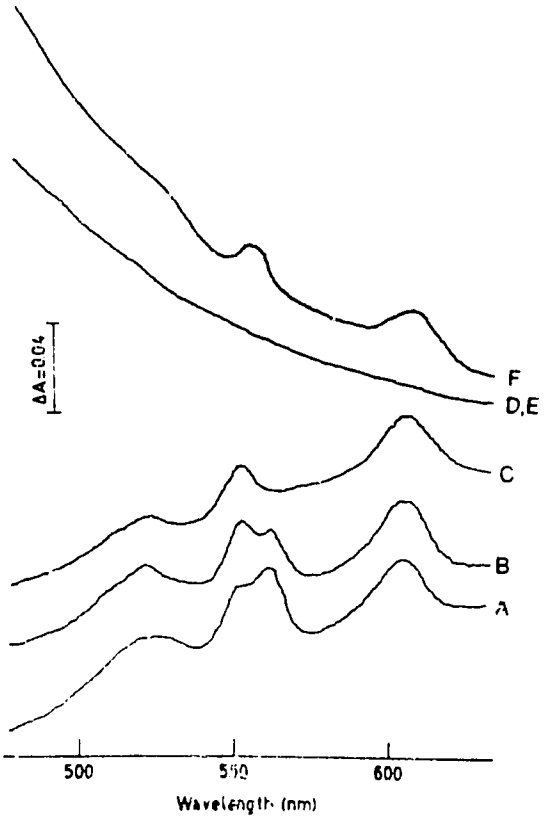

Fig. 4. Difference spectra (reduced *minus* oxidized) of SMP under various conditions. Traces shown correspond to the difference between spectra of SMP (1.9 mg protein/ml) treated as explained below and untreated oxidized SMP. The reaction medium (1.5 ml) was 50 mM potassium phosphate (pH 7.5). Treatments were as follows: (A) $Na_2S_2O_4$, (B) 10 mM succinate, (C) 5 mM ascorbate + 0.1 mM TMPD, (D) incubation of SMP with 20 mM DIMBOA for 15 min and further addition of 10 mM succinate, (E) incubation of SMP with 20 mM DIMBOA for 15 min, without addition of reducing agents, and (F) incubation of SMP with 20 mM DIMBOA for 15 min and further addition of 5 mM ascorbate + 0.1 mM TMPD.

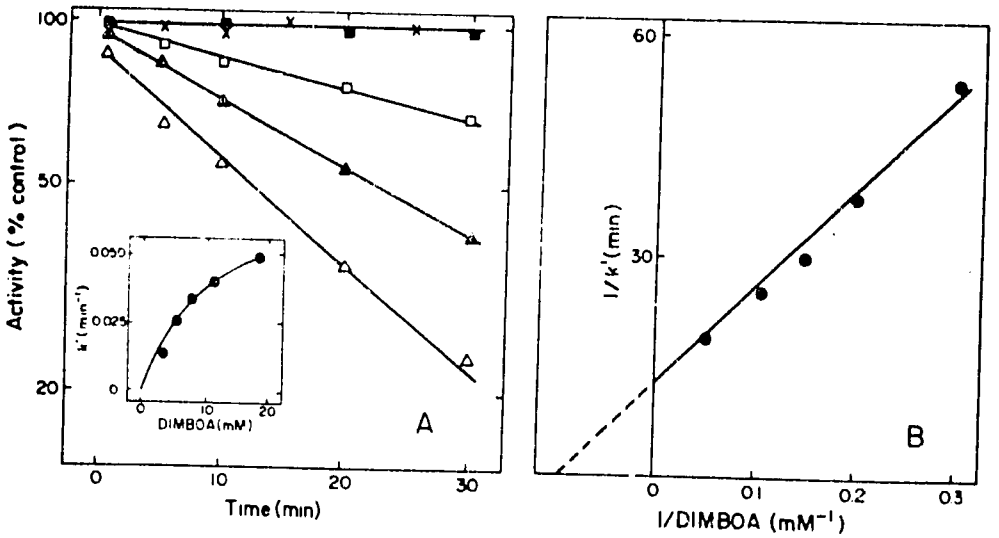

Fig. 5. Irreversible inactivation of P_i-ATP exchange reaction. Experimental conditions were similar to those explained in the legend to Fig. 2 except that in this case P_i-ATP exchange reaction was measured (see Materials and Methods). DIMBOA concentrations were: 0 (■), 3 (□), 7 (▲) and 18 mM (△). (×) corresponds to SMP treated with 20 mM DIMBOA and afterwards assayed for ATPase activity as indicated under Materials and Methods. In the inset are plotted the pseudo-first-order rate constants (k') determined from the slopes of the semilogarithmic plots shown. Activity for control was 98 nmoles/min · mg protein.

Preincubation of SMP with DIMBOA also provoked an exponential inactivation of the P_i-ATP exchange reaction (Fig. 5A). The pseudo-first-order rate constants, in this case, were dependent in a hyperbolic manner on DIMBOA concentration (Fig. 5A, inset) since the double-reciprocal plot of k' versus DIMBOA concentration yielded a straight line that intersected the y-axis at a value significantly different from zero (Fig. 5B). Conversely, when ATPase activity was measured with DIMBOA-treated SMP, no inactivation could be observed (see Fig. 5A).

DISCUSSION

DIMBOA exerted several actions on mitochondrial energy metabolism. At concentrations above 5 mM it inhibited electron transport from NADH, succinate and ascorbate + TMPD to oxygen, acting at the level of the respiratory chain since similar results were obtained in the presence of uncouplers (data not shown). These results suggest that the site of action of the inhibitor can be tentatively located in the cytochrome chain after the site of entry of electrons from ascorbate + TMPD. Since it was not possible to observe an instantaneous change in the redox state of succinate-reduced SMP by addition of DIMBOA in concentrations inhibiting electron transport more than 80% (data not shown), it can be suggested that the reversible inhibition of electron transport is produced between cytochrome *a* and O_2.

At lower concentrations, DIMBOA inhibited ATP synthesis, P_i-ATP exchange reaction and ATPase activity. These effects cannot be attributed to the action of DIMBOA on electron transport and are probably exerted at the mitochondrial ATPase complex, since the ATPase activity was also inhibited by DIMBOA. This inhibition was reversible since a 100-fold dilution restored the original activity.

When these effects are compared to the reversible effects described on ATP synthesis in chloroplasts [9], several differences can be pointed out: (i) in chloroplasts DIMBOA behaved exclusively as an energy transfer inhibitor whereas in SMP it also behaved as an electron transport inhibitor, and (ii) chloroplasts were twice as sensitive to DIMBOA as SMP.

A second category of effects probably related with the known reactivity of DIMBOA (see Refs. 11 and 13) resulted in the irreversible inactivation of some mitochondrial functions. Electron transport either from succinate or from NADH to oxygen were similarly inactivated upon incubation of SMP with DIMBOA. The inactivation followed pseudo-first-order kinetics, suggesting that the activity is suppressed after reaction of one group in the enzymatic complex with DIMBOA. From the kinetic data it can be postulated that DIMBOA reacted with some component in the respiratory chain following a simple second-order reaction and resulting in an irreversible inactivation of mitochondrial electron transport (see reaction scheme I in Fig. 6). From the inset in Fig. 2 the second-order rate constant (k) can be estimated to be equal to 4.4 M^{-1} min^{-1}. The electron transport chain component modified by DIMBOA is probably located at the level of the cytochrome chain since: (i) NADH and succinate oxidation were equally inactivated, and (ii) succinate was able to completely protect both reactions against inactivation by DIMBOA (see Fig. 3). The protection by succinate cannot be explained by binding of succinate to succinate dehydrogenase since this enzyme is not in the path of electrons from NADH to oxygen and hence is unlikely to be the electron

I

$$E + DIMBOA \xrightarrow{k} E_i$$

II

$$E + DIMBOA \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot DIMBOA \xrightarrow{k_2} E_i$$

Fig. 6. Irreversible inactivation of mitochondrial reactions by DIMBOA. E represents the unmodified fully active enzyme form. E_i represents an inactive form, either an adduct of the enzyme and DIMBOA or an irreversibly modified enzyme form. E·DIMBOA represents a reversible intermediate whose dissociation constant (K_i) equals k_{-1}/k_1.

transport chain component affected by DIMBOA. Therefore, succinate protection must be exerted in an indirect way probably by changes in the redox state of some electron transport chain component located in the cytochrome chain near the point of entry of electrons from succinate dehydrogenase. The change in redox state of such a component could be accompanied by changes in nucleophilicity and/or reducing power [11, 13] of the groups involved in the irreversible modification. Further experimental evidence allowed the location of the site of action of DIMBOA as an irreversible inhibitor of the respiratory chain: (i) ascorbate + TMPD oxidation was not inactivated upon incubation of SMP for 30 min with 20 mM DIMBOA and (ii) cytochromes *b*, *c* and *a* cannot be reduced by succinate in DIMBOA-treated SMP (Fig. 4, spectrum D), whereas cytochromes *c* and *a* can be reduced by ascorbate + TMPD (Fig. 4, spectrum F). Therefore, the site of inactivation by DIMBOA must be located before cytochrome *c* and after succinate dehydrogenase (see above). Whether the appearance of a chromophore upon incubation of SMP with DIMBOA (Fig. 4, spectrum E) is related to the inactivation of electron transport by DIMBOA is still a matter of study.

The inactivation of the P_i-ATP exchange reaction cannot be explained by reaction scheme I, since the hyperbolic dependence of the pseudo-first-order rate constants with DIMBOA concentration indicates that a non-covalent intermediate or an easily-reversible covalent intermediate is formed prior to the irreversible modification (see reaction scheme II in Fig. 6). From the minus reciprocal of the x-intercept in Fig. 5B the dissociation constant (K_i) for the reversible intermediate can be estimated to be equal to 7.5 mM and, from the minus reciprocal of the y-intercept in the same figure, the value of k_2 (rate constant for the irreversible step) can be estimated as equal to 0.1 min^{-1}. The site modified by DIMBOA that is responsible for the P_i-ATP exchange inactivation must necessarily be different from the one responsible for electron transport inactivation, since both inactivations followed kinetically different reaction schemes.

An interesting point is that the ATPase activity of SMP was not inactivated at all by DIMBOA, indicating that the mitochondrial ATPase complex was not modified or that it was modified in such a way that the enzyme complex was still able to hydrolyze ATP. However, this ATP hydrolysis was not able to build a proton gradient through the mitochondrial inner membrane since the P_i-ATP exchange reaction and the ATP-dependent ANS fluorescence enhancement (data not shown) were inactivated completely when SMP were preincubated for 30 min with 20 mM DIMBOA. The inactivation of these two energy-linked (ATP-driven) reactions can be explained by an irreversible uncoupling of ATP hydrolysis and proton gradient build-up whose mechanism is still a matter of study.

An additional difference between the effect of DIMBOA on mitochondria and on chloroplasts is that ATPase activity in chloroplasts was irreversibly inactivated by DIMBOA whereas mitochondrial ATPase activity was not. This difference is probably related to the fact that, in the β-subunit of chloroplast coupling factor CF_1, there are essential cysteine residues (see Refs. 10, 11 and 13). Conversely, it has been recently confirmed that β-subunit from mitochondrial ATPase does not contain cysteine residues [24].

In summary, the actions of DIMBOA on SMP, described in this paper, suggest that mitochondrial metabolism is a good candidate for explaining the biological action of these hydroxamic acids on organisms that depend largely on mitochondrial ATP synthesis. From the point of view of the toxicity of DIMBOA, probably its most relevant effects on mitochondrial energy metabolism were the irreversible inactivation of electron transport and the irreversible uncoupling of the ATP-driven energy-linked reactions. Either effect would produce an inactivation of mitochondrial ATP synthesis leading to increased ineffectiveness of the energy metabolism of aerobic organisms.

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A NEW PRODUCT FROM THE DECOMPOSITION OF 2,4-DIHYDROXY-7-METHOXY-1,4-BENZOXAZIN-3-ONE (DIMBOA), A HYDROXAMIC ACID FROM CEREALS

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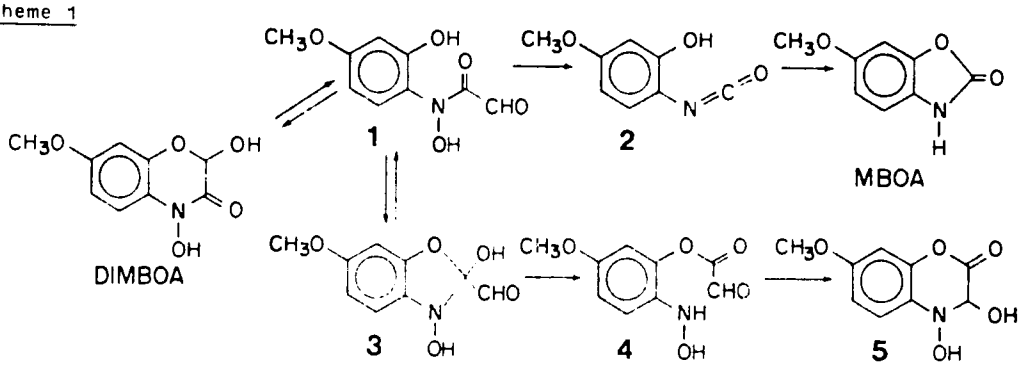
Abstract- A new product, identified as 3,4-dihydroxy-7-methoxy-1,4-benzoxazin-2-one (5) was isolated in 10% yield from the decomposition of the title compound in dioxane. A mechanism for its formation is discussed.

Hydroxamic acids from cereal extracts, such as the title compound, DIMBOA, play an important defensive role in the plant.[1,2] In interpreting the mode of action of DIMBOA at a molecular level, its chemistry in solution, in particular its decomposition reaction, has been of paramount importance.[3] The reaction has been shown to give, as main product, 6-methoxy-benzoxazolin-2-one (MBOA), through the formation of the isocyanate 2 (Scheme 1).[4,5] The yield of MBOA is not quantitative.[6] We report herein the structure of a secondary product of the reaction, 5.

Scheme 1



The yield of MBOA from the decomposition of DIMBOA was dependent upon the basicity of the solvent system employed. In aprotic solvents, the logarithm of the yield correlated linearly (r= 0.940) with solvent donor number, a measure of the ability of the solvent to donate an electron pair.[7] In aqueous solutions, the logarithm of the yield correlated linearly (r= 0.996) with pH (Fig. 1).

These results are consistent with the mechanism shown in Scheme 1. Thus, an increase in nucleophilicity of the hydroxamic hydroxyl group, either by interaction with an electron-donating solvent or by dissociation, would favor its attack on the aldehyde group of 1 with formation of intermediate 2.

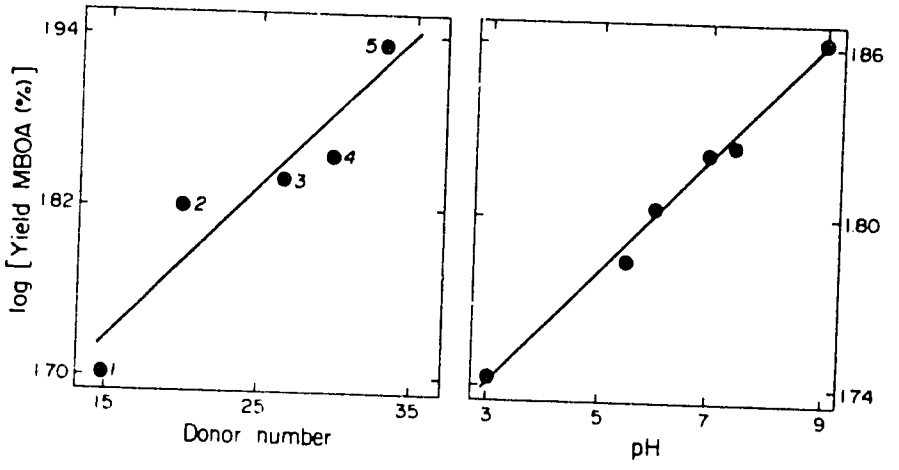


Fig. 1. Effect of donor number and pH on the yield of MBOA from the decomposition of DIMBOA in aprotic and aqueous solvents, respectively. DIMBOA was decomposed for at least 5 half-lives. Dioxane= 1, tetrahydrofuran= 2, N,N-dimethylformamide= 3, dimethylsulfoxide= 4, pyridine= 5.

Attempts to isolate secondary products were carried out in dioxane, the solvent producing the lowest yield of MBOA. DIMBOA was decomposed in dioxane at 63°C for 2 weeks. The residue was subjected to dry column flash chromatography and then to thin layer chromatography. A product with R_f= 0.43 (silica gel GF_{254} with chloroform:methanol 10:1 as developing solvent), isolated in 10% yield, had the following properties: UV λ_{max} (EtOH) nm: 257, 287sh; λ_{max} (H_2O, pH 7) nm: 259 (ϵ= 15,400), 285sh; λ_{max} (H_2O, pH 13) nm: 255sh, 300 (ϵ= 10,900); pK_a= 11.3; IR ν_{max} (KBr) cm^{-1}: 3200, 2950, 1665, 1610, 1500, 1460, 1280, 1140, 1080, 1020, 820; ^{1}H-NMR (60 MHz, CD_3OD)δ: 3.8 (3H,s,CH_3O), 5.5 (1H,s,CHOH), 6.4-7.0 (3H,m,aromatic); EIMS (probe, 70 eV) m/z (rel. int.): 211 (72)$[M]^+$, 180 (100) $[M-OCH_3]^+$, 165 (39) $[180-OH]^+$, 153 (37) $[M-CO-CHOH]^+$, 137 (48) $[153-O]^+$, 122 (40) $[153-NOH]^+$, 106 (21) $[122-O]^+$; negative $FeCl_3$ test; positive Tollens' test. This data is consistent with structure 5, an isomer of DIMBOA. This product could be detected by thin layer chromatography in the decomposition mixture of DIMBOA in the other aprotic solvents studied.

The formation of 5 may be rationalized as shown in Scheme 1. The open form of DIMBOA, 1, may be demonstrated and quantified polarographically in aprotic solvents.[8] Reclosure to hemiketal 3 has been proposed in the reactions of related compounds.[9] Opening of 3 would follow to form 4 which would finally cyclize to yield 5 by addition of a hydroxylamine nitrogen to a carbonyl group, a reaction analogous to the well-known synthesis of hydroxamic acids.

The formation of 5 provides a rationalization of hitherto unexplained facts in the literature. Thus, the decrease of the yield of MBOA from DIMBOA as pH is lowered may be attributed to acid catalysis of the conversion of 3 to 4. Furthermore, the decomposition in strong acid of these hydroxamic acids has been reported to yield the corresponding 2-hydroxy-phenylhydroxylamine.[10] Hydrolysis of ester 4 accounts for this product.

Other products from the reaction studied were coloured, resinous substances, which likely arise from oxidation and condensation of these intermediates.[11]

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Effect of gramine in the resistance of barley seedlings to the aphid *Rhopalosiphum padi*

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Keywords: Gramineae, barley, *Hordeum*, Aphid, *Rhopalosiphum padi*, *Schizaphis graminum*, greenbug, alkaloid, gramine

Abstract

Gramine (N,N-Dimethyl-3-aminomethylindole) content in various barley cultivars varied from 0 to 2.6 mmoles/kg fresh weight. Those cultivars which were lacking gramine were the most susceptible to the aphid *Rhopalosiphum padi* (L.). The population growth rate of *R. padi* negatively correlated with gramine content in leaves of barley seedlings. In addition, gramine incorporated in artificial diets decreased survival, amount of diet ingested and reproduction of aphids at concentrations similar to those found in plant leaves. Thus, it is suggested that gramine may be one of the factors responsible for the resistance of barley seedlings to *R. padi*.

Introduction

Secondary compounds may protect plants against herbivores. For example, acyanogenic morphs of *Lotus corniculatus* L. and *Trifolium repens* L. are preferred over the cyanogenic morphs by a variety of invertebrate and vertebrate herbivores (Rhoades, 1979). Sheep avoid grazing on varieties of lupine that contain high concentrations of alkaloids but readily graze on 'sweet' varieties (Arnold & Hill, 1972). The resistance of some varieties of barley to aphids can be attributed to the presence of several phenolic and flavonoid compounds (Todd *et al.*, 1971). Gramine and related indole alkaloids present in Gramineae, Leguminosae and other families (Saxton, 1965) are responsible for their toxicity to ruminants (Gallagher *et al.*, 1964). Indole alkaloids were also reported as toxic to aphids (Corcuera, 1984). In addition, Zúñiga *et al.* (1985) have reported the role of indole alkaloids as a factor in the resistance of several barley (*Hordeum*) cultivars to the greenbug *Schizaphis graminum* (Rondani). In this paper we examined the effect of gramine contained in barley to the aphid *Rhopalosiphum padi*.

Materials and methods

Aphids. Individuals of *R. padi* used for the experiments were collected from naturally infested barley near Santiago. Aphid colonies were maintained on *Hordeum distichum* cv 'F. Union' grown under continuous light in the laboratory.

Infestation experiments. Seeds of five different barley cultivars, namely cv F. Union, Abyssinian, comun, CM67-Galt and Brea 'S' Celaya, were planted in pots filled with soil and grown under continuous light in a greenhouse at 23–28 °C. Ten-day-old plants were infested with two aphids, apterous gynoparae, and the increase in the insect population was determined after 6 days. Gramine content of leaves was also determined in 10- and 16-day-old seedlings. Three samples of five seedlings each were used for each treatment.

Preparation of extracts for gramine analyses. The samples (2 to 3 g of leaves) for the alkaloid analyses were frozen and macerated using mortar and pestle with 20 ml MeOH-NH_4OH (100:1 v/v). The extract was filtered through glasswool. The solvent and en-

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dogenous water were evaporated under an air stream. The chlorophyllous residue was dissolved in 5 ml 0.1 N HCl and filtered through Whatman No. 1 paper. The pH of aqueous filtrate was adjusted to pH 9 with conc NH_4OH and washed twice with chloroform (1:2 v/v). The organic phase was evaporated to dryness. Gramine was quantified in these extracts as described previously (Zúñiga *et al.*, 1985).

Feeding assays. Aphid feeding assays were made by using holidic diets placed between two layers of Parafilm M (Argandoña *et al.*, 1982). The diet consisted of a solution containing 35% sucrose, aminoacids and mineral salts, adjusted to pH 6 (Corcuera, 1984). The amout of diet ingested by ten aphids was determined by weighing the Parafilm sachets before and after 7 h of feeding. Three samples of ten aphids each were used for each treatment.

Each point in Fig. 2 represents the average of three samples of ten adults aphids each.

Results and discussion

Gramine content and resistance to aphids. Seedlings of the five barley cultivars were selected for infestation experiments with *Rhopalosiphum padi*. The most susceptible variety was 'F. Union', which lacked gramine, while the varieties with higher gramine contents were more resistant to *R. padi* (Fig. 1). Similar results have been reported with *Schizaphis graminum* (Zúñiga *et al.*, 1985). However, the seedlings with more gramine were more resistant to *R. padi* than to *S. graminum*.

Effect of gramine on aphids. Incorporation of gramine into artificial diets decreased survival of *R. padi* at all concentrations tested (Fig. 2). To determine if gramine acted as a feeding deterrent aphid nymphs were fed on diets containing 0 to 6 mM gramine for 7 h. The amount of diet ingested by the aphids decreased with gramine content (Fig. 3A). After 7 h of feeding with diets with various gramine concentrations, the aphids were transferred to control diets without gramine. Survival was determined 48 h later. Survival of aphids at 0 and 6 mM were the highest, while the lowest survival was observed at intermediate gramine concentrations (Fig. 3B).

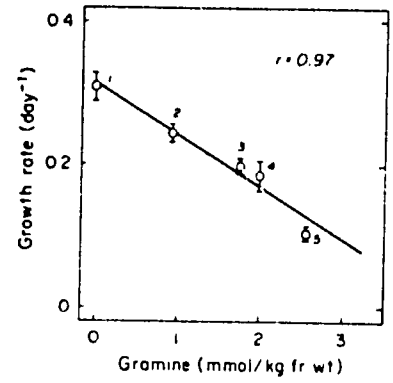

Fig. 1. Gramine content of leaves and population growth rate of *R. padi* on barley seedlings. Numbers of Barley cultivars: 1 = 'F. Union', 2 = 'CM67-Galt', 3 = 'BREA "S"-Celaya', 4 = 'comun', 5 = 'Abyssinian'. Vertical bars: ±1 s.e. Aphid population growth rate = Ln (Nf/Ni)/Δt.

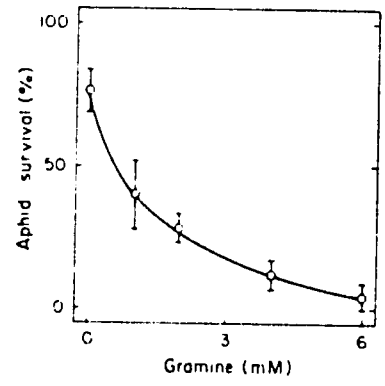

Fig. 2. Effect of gramine on the survival of adults after 48 h feeding of *R. padi* on diets containing various gramine concentrations. Vertical bars: ±1 s.e.

As indicated in Fig. 3A, the highest survival of aphids exposed to the 6 mM gramine diet was probably due to non-feeding. When the diet was ingested, a higher mortality was observed. Thus, gramine causes toxic effects and also feeding deterrency. In addition, reproduction rates of aphids feeding on artificial diets decreased even at relatively low concentrations of gramine (Fig. 4). These low concentrations were selected for the experiment because feeding deterrency was also low and gramine containing diets were ingested in similar amounts as the control diet.

It is likely that gramine plays a role in the resistance of barley against aphid attack. Although, the

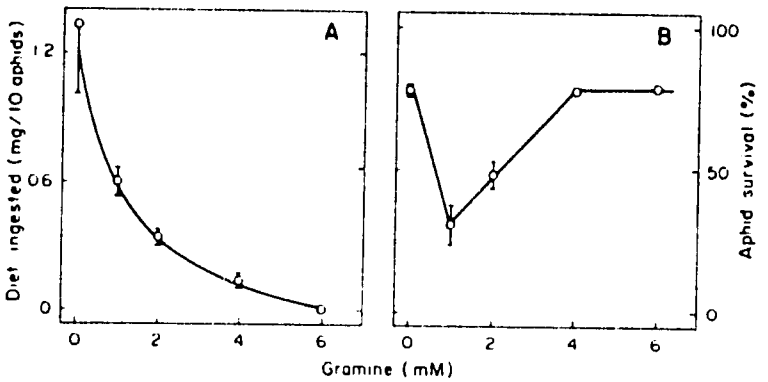

Fig. 3. Feeding deterrency and toxicity of gramine. After the nymphs were exposed for 7 h to the diets, ingestion was determined by weighing the sachets (A). Then they were transferred to control diets, and survival was determined after 48 h (B). Vertical bars: ±1 s.e.

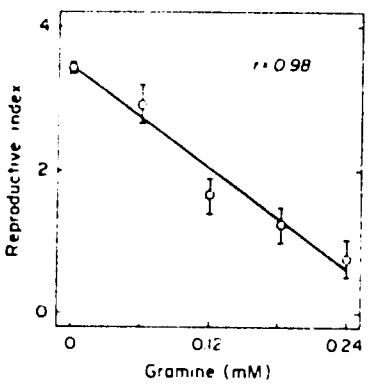

Fig. 4. Effect of gramine on the reproduction of *R. padi* reared on artificial diet. The reproductive index (no. nymphs/av. no. adults) was measured after feeding adults for 72 h. Vertical bars: ±1 s.e.

exact location of gramine in the leaves is unknown, the feeding deterrent and toxic properties of this compound appeared to be relevant to plant protection.

Barley is an important grazing crop in warm, dry areas. Development of gramine-free barley cultivars has been suggested as desirable because of the use of this crop for animal feeding (Hanson *et al.*, 1981). However, this paper and our previous work (Corcuera, 1984; Zúñiga *et al.*, 1985) have shown that barley cultivars which contain a low amount of gramine are more susceptible to insect attack than those cultivars with a high gramine content. Thus, eliminating gramine from barley by means of plant breeding would be advisable only for those cultivars that have alternative insect resistance mechanisms.

Acknowledgements

Supported by Agency for International Development, Universidad de Chile (Project N-1654), and Fondo Nacional de Desarrollo Científico y Tecnológico (Project No. 1035). We thank Ing. Agr. Edmundo Beratto for providing the seeds of the barley cultivars.

Résumé

Effet de la gramine sur la résistance de plantules de seigle des puceron Rhopalosiphum padi

La teneur de gramine (N,N-dimethyl-3-aminomethyl-indole) dans différentes cultures de seigle présente des variations comprises entre 0 et 2,8 mmoles/Kg (poids frois). Les varietés dépourvues de gramine sont plus sensibles à l'attaque des pucerons. Le taux de croissance de la population des *Rhopalosiphum padi* a une correlation négative avec la teneur en gramine des feuilles de plantules de seigle. D'ailleurs, la gramine diminue les taux de nourrissement, de survie et de réproduction des pucerons alimentés avec des diètes artificielles contenant des concentrations du produit testé, similaire à celles trouvées pour les feuilles des plantes. Donc, on suggèrent que la gramine peut être un des facteurs responsables de la résistance des plantules de seigle contre l'attaque de *Rhopalosiphum padi*.

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Effects of hydroxamic acids on the resistance of wheat to the aphid *Sitobion avenae*

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SUMMARY

Resistance to the aphid *Sitobion avenae* F. was assessed on six wheat cultivars of known concentrations of total hydroxamic acids. A range of values for intrinsic rate of increase (r_m) were obtained, 96% of the variation in which was explained by acid concentrations. Of the components of r_m, age-specific fecundity was the most significant factor contributing to resistance.

INTRODUCTION

Sitobion avenae F. is a sporadically damaging pest of wheat in temperate climates (George & Gair, 1979; Vickerman & Wratten, 1979; Carter, McLean, Watt & Dixon, 1980). Wheat extracts contain hydroxamic acids (Hx) (Willard & Penner, 1976) which have been shown to be important in resistance against insects in several Gramineae (Klun, Tipton & Brindley, 1967; Klun, Guthrie, Hallauer & Russell, 1970; Long, Dunn, Bowman & Routley, 1977; Beck, Dunn, Routley & Bowman, 1983). The most abundant of these acids in wheat is 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA). This compound has also been shown to be involved in the resistance of several wheat cultivars to the aphid species *Metopolophium dirhodum* (Wlk.), *Schizaphis graminum* (Rond.) and *Rhopalosiphum maidis* (Fitch) (Corcuera, Argandoña & Niemeyer, 1982), but *S. avenae* has not been investigated in this context.

The main objective of this investigation was to assess a range of wheat cultivars, previously assessed for hydroxamic acid levels, in order to measure and rank them for antibiotic resistance to *S. avenae*. Any correlations between aphid performance and acid levels would have implications for future screening of wheat material against this important European pest.

MATERIALS AND METHODS

Seed samples of six wheat cultivars representing a range of known Hx levels were grown under permanent light at *c.* 26 °C with a 10 °C range in a glasshouse at the University of Chile, and harvested at the two-leaf stage (G.S. 12; Zadoks, Chang & Konzak, 1974). A portion, 1·5 g, of the plant tissue (10–15 plants) was then macerated with a mortar and pestle in water (6 ml total volume), filtered through cheesecloth and left for 15 min at room temperature. The extract was adjusted to pH 3 with 1 M HCl and centrifuged at 10 000 *g* for 10 min. The supernatant was extracted three times into equal volumes of ethyl ether and the organic phases were evaporated to dryness. Hx form a blue complex (λ_{max} = 590 nm) upon the addition of ferric chloride reagent (50 g $FeFl_3.6H_2O$, 500 ml 95% ethanol and 5 ml 15 M HCl). The concentration of Hx in the tissues was determined by comparing the absorbance of extracts

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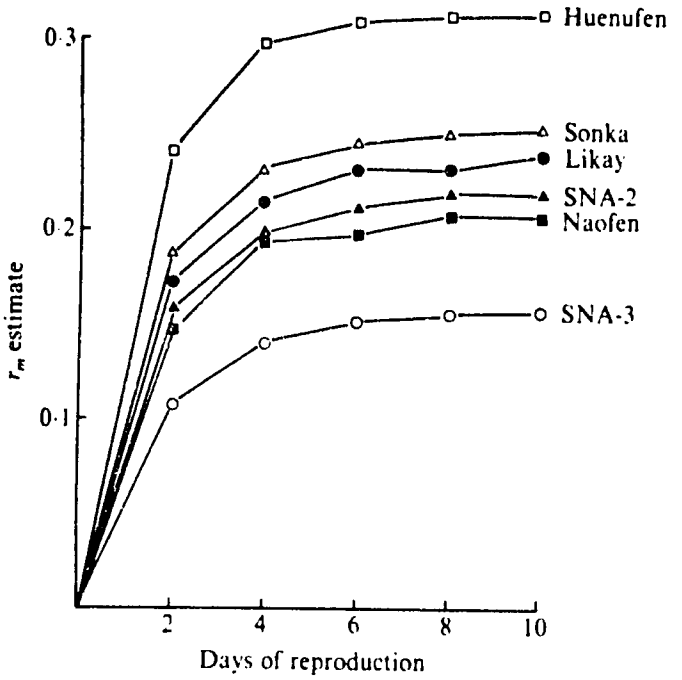


Fig. 1. Effect of assessment period on the estimate of intrinsic rate of natural increase (r_m) of *Sitobion avenae* on six wheat cultivars.

with a standard curve made with DIMBOA. The procedure was tested by adding DIMBOA to an Hx-lacking barley cultivar (cv. Fola Union – see Argandoña, Luza, Niemeyer & Corcuera, 1980). Recovery of DIMBOA was 100%. DIMBOA had previously been isolated from 7-day old seedlings of maize (cv. T 129S) by the method of Queirolo, Andreo, Niemeyer & Corcuera (1983). The names of the cultivars assessed are given in Fig. 1.

For assessment of aphid performance at Southampton, seeds of the same cultivars were planted singly in 5-cm diam. polystyrene pots containing John Innes No. 2 seed compost and were germinated in a glass-house at a temperature of 20 °C with an 8 °C range and a minimum 16 h photoperiod. They were transferred after *c.* 5 days to a culture room based on the design of Scopes, Randall & Biggerstaff (1975). They were kept at 20 °C with a 2 °C range, 60–70% r.h. and 16 h photoperiod. Light intensity was 75 μE m^{-2} s^{-1}.

Stock cultures of *S. avenae* were clonal, originating from a single parthogenetic female collected in Hampshire, UK, in 1984 and were maintained on wheat (cv. Hobbit) in the culture room under the above conditions. Adult apterous viviparae of unknown age were placed individually on the test seedlings (30 of each cultivar) which were at the two-leaf stage; these were then covered with transparent plastic cylinders 20 cm high with a Terylene mesh top. These aphids were left for 24 h to reproduce and then removed together with all but two first-instar nymphs. The latter were left undisturbed until they moulted to the adult stage, but were checked daily during this period. One was them removed and the daily fecundity of the remaining singly-caged aphids (up to 30 on each cultivar) was recorded for 10 days. Progeny of these were removed daily, using a fine paint brush, during the same 2 h period each day.

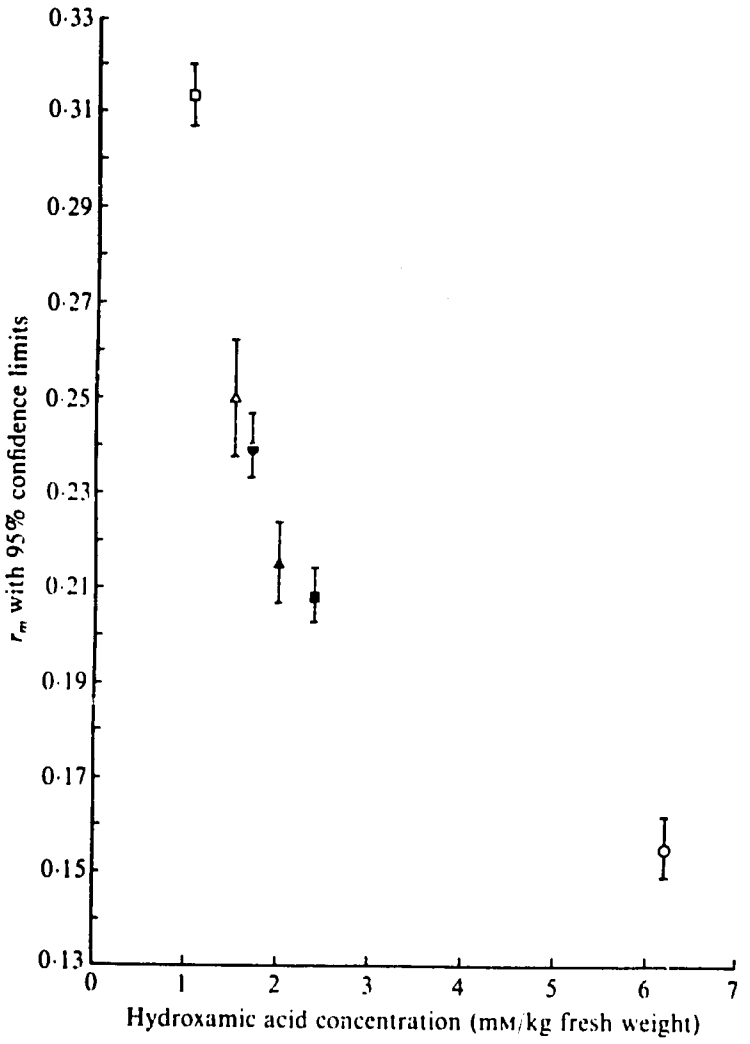
The intrinsic rate of natural increase (r_m; Birch, 1948) was calculated for the aphids on each cultivar using a program incorporating the 'Jack-knife' technique to calculate the standard error (Miller, 1974; Bissell, 1977; Birch & Wratten, 1984). Age-specific survival (l_x), and age-specific fecundity (m_x) were also computed.

RESULTS AND DISCUSSION

Estimates of r_m were calculated at daily intervals. On all cultivars, nymph production during the first few days of reproduction contributed most to the value of r_m (Fig. 1), a pattern similar to those found for other aphid species (e.g. Wyatt & White, 1977; Birch & Wratten, 1984). Values of r_m based on 10 days' recording were used in subsequent analysis.

There was a highly-significant negative relationship between the r_m value achieved on the cultivars and the concentration of hydroxamic acids in their tissues; the proportion of the variation in r_m values explained by acid levels was up to 96%, depending on whether the variables were arithmetic (Fig. 2*a*), or one, or both (Fig. 2*b*) variables, were logarithmically transformed. The confidence limits for r_m were very low; however, the parallel inter-plant chemical variation could not be calculated because of the bulking of samples needed to provide 1·5 g of plant tissue (see Methods).

Of the components of r_m, mortality did not begin on any cultivar until 16 days after birth (Fig. 3); this was well after the highest daily fecundity had been reached (8–12 days after birth). This pattern agrees with that found by Frazer (1972) for two aphid species on *Vicia faba* L. cultivars. In other work, where a wider taxonomic range of plant material has been



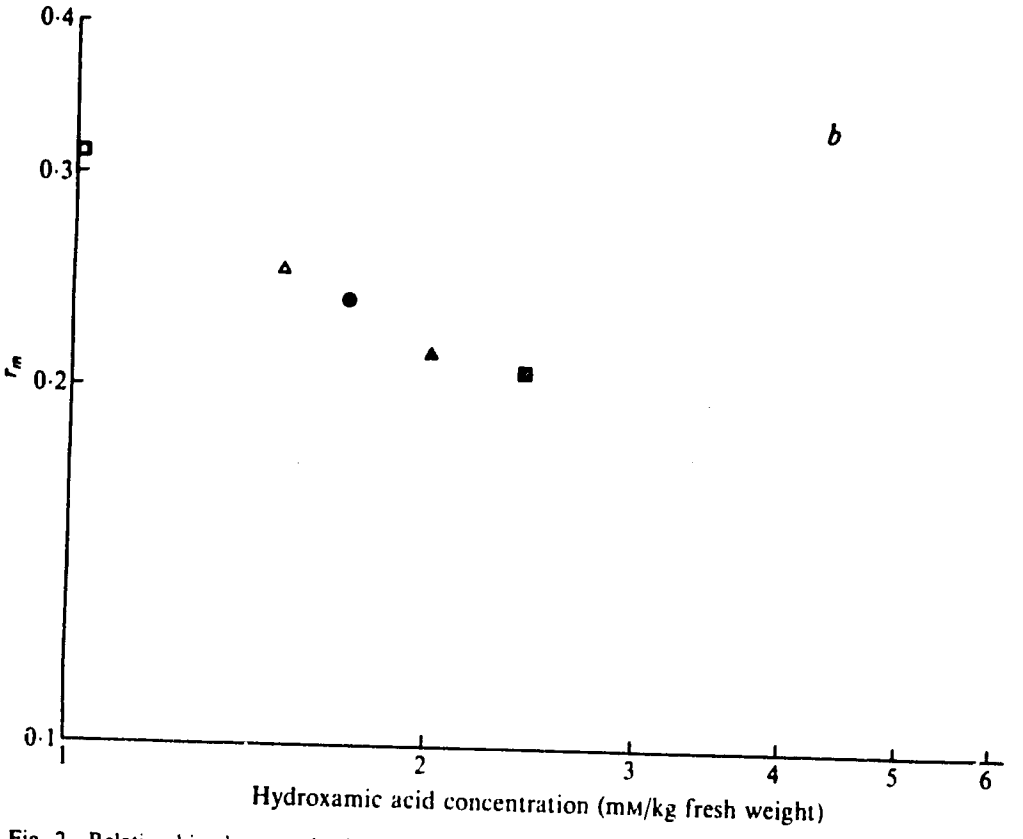


Fig. 2. Relationships between intrinsic rate of natural increase of *Sitobion avenae* and levels of total hydroxamic acids in wheat seedlings at the two-three-leaf stage. *a*. arithmetical (with 95% confidence limits); *b*. logarithmic; log y = −1·22 − 0·37 log b. $S.E._b$ = 0·033, $P < 0·001$, r = −0·98, $P < 0·001$. Symbols as in Fig. 1.

screened, extremes of survival and reproductive performance occur (e.g. Birch & Wratten, 1984).

In addition to the correlation in Fig. 2*b*, the age of the aphids at the time of the peak in age-specific fecundity was positively related to acid levels (log y = 2·17 + 0·17 log x; $P < 0·05$; r = 0·86).

This preliminary investigation has revealed that hydroxamic acids appear to play a major role in seedling resistance to *Sitobion avenae*. Future work must include a wider taxonomic range of *Triticum* material, which should be screened for aphid resistance and hydroxamic acid levels, ideally in the same plant cohorts at a range of growth stages. Although Argandoña *et al.* (1980, 1981) demonstrated declines in acid levels with increasing plant age, the rate of this change needs to be compared within a wider range of taxa, with a view to selecting those with the lowest rates. Even for those *Triticum* cultivars and species with high acid levels only at the seedling stage, useful resistance potential remains; dipterous pests of the genera *Oscinella* and *Opomyza* attack seedlings in the field in Europe and the aphids *Rhopalosiphum padi* (L.) and *S. avenae* colonise seedling cereals in the autumn in the UK, when plant pathogenic viruses may also be transmitted.

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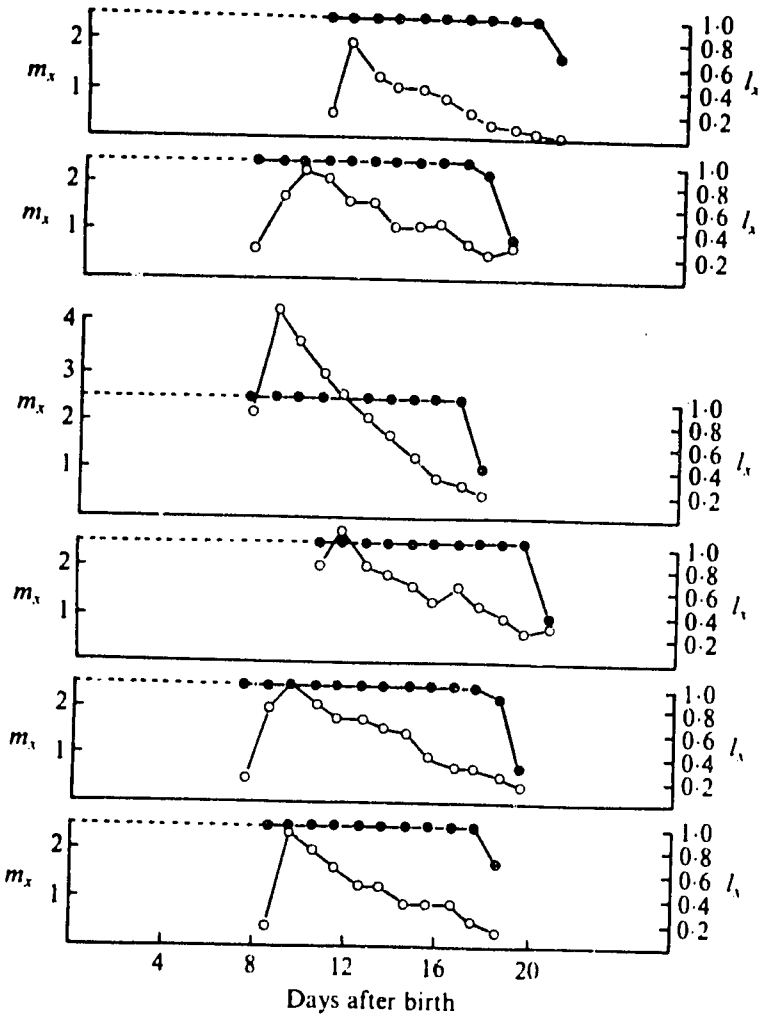

Fig. 3. Changes in age-specific fecundity (m_x, ○) and age-specific survival (l_x, ●) for the six cultivars (top to bottom): SNA-3, Likay, Huenufen, Naofen, Sonka, SNA-2.

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Quantitation of *N*-(2-Hydroxy-4-methoxyphenyl)glyoxylohydroxamic Acid, a Reactive Intermediate in Reactions of 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one

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Extracts of various Gramineae contain hydroxamic acids such 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), which are involved in the resistance of the plants to pests and pathogens.[1,2] It was suggested that the toxicity of DIMBOA is partly due to reactions of its open-chain isomer, *N*-(2-hydroxy-4-methoxyphenyl)glyoxylohydroxamic acid (1).[3,4] On the basis of rate and product studies,[5-7] this intermediate has been invoked in the decomposition reaction of DIMBOA. Since 1 has electroactive groups different from those of DIMBOA, we attempted its quantitation by polarographic reduction of

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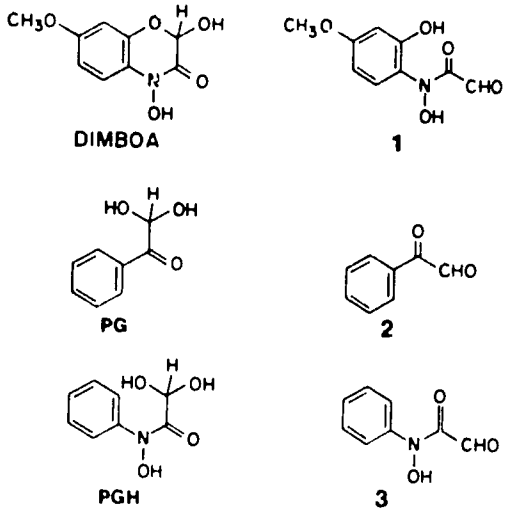

solutions of DIMBOA. We describe herein the quantitation of 1 in aprotic solvents and discuss its reactivity and the mechanism of decomposition of DIMBOA.

Experimental Section

DIMBOA was isolated as described[8] from ethereal extracts of 6-day old seedlings of *Zea mays* L. cv T129s grown in a greenhouse at 25 ± 5 °C. Phenylglyoxal monohydrate (Aldrich) was used without further purification. Solvents were purified and dried by described methods[9] and were stored under nitrogen.

Polarograms were taken with a PAR Model 174A polarograph on DIMBOA solutions thermostated at 28 °C. The cell, designed for measurements under inert atmosphere, contained a dropping mercury electrode (DME) as working electrode, a platinum auxiliary electrode, and a standard calomel electrode (SCE) as reference electrode. Dry argon was bubbled through the solutions for 10 min before each experiment. Runs were carried out with the solvents in the absence of DIMBOA to test for oxygen leaks. No leaks were detected even after successive potential scans for more than 5 h. The supporting electrolyte was 0.1 M tetraethylammonium perchlorate.

Synthesis of *N*-Phenyl-α,α-dichloroacetohydroxamic Acid. To a solution of 0.035 mol of freshly prepared phenylhydroxylamine in 45 mL of anhydrous diisopropyl ether was added an excess of sodium bicarbonate. To this suspension was added, under constant stirring, 0.018 mol of dichloroacetyl chloride dissolved in 5 mL of diisopropyl ether, with the temperature kept between 0 and 5 °C. After 30 min the mixture was filtered and the filtrate concentrated to turbidity. Petroleum ether was added to complete precipitation. The residue was filtered and recrystallized from benzene–petroleum ether (yield, 93%): mp (uncorrected) 91 °C; UV λ_{max}^{EtOH} 263 nm; IR ν_{max}^{KBr} (cm^{-1}) 3200, 1635; EIMS (probe, 70 eV), *m/z* (relative intensity) 220 (5, $[M]^+$), 136 (32, $[M - CHCl_2]^+$), 108 (100, $[136 - CO]^+$); ^{1}NMR (60 MHz, CD_3COCD_3) δ 7.1 (1 H, s, $CHCl_2$), 7.2–7.9 (5 H, m, Ar); positive $FeCl_3$ test. Anal. Calcd for $C_8H_7NO_2Cl_2$: C, 43.67; H, 3.21; Cl, 32.22. Found: C, 43.89; H, 2.97; Cl, 32.61.

Synthesis of *N*-Phenylglyoxylohydroxamic Acid Monohydrate (PGH). This compound was obtained by hydrolysis of *N*-phenyl-α,α-dichloroacetohydroxamic acid in 1.5 N NaOH at room temperature for 2 h. The resulting solution was extracted with diethyl ether and then acidified to pH 3 with concentrated HCl. The acidified aqueous solution was extracted with diethyl ether. The organic phase was dried with Na_2SO_4 and evaporated under vacuum. The orange oil left was treated with benzene, upon which white crystals were obtained. These crystals were further washed with benzene. The yield was less than 10%: mp (uncorrected) 155–157 °C; UV λ_{max}^{EtOH} 253 nm, $\lambda_{max}^{H_2O}$ 244 nm (ε 14 800); IR ν_{max}^{KBr} (cm^{-1}) 3360, 1665; EIMS (probe, 70 eV), *m/z* (relative intensity) 183 (31, $[M]^+$), 165 (100, $[M - H_2O]^+$), 136 (50, $[165 - CHO]^+$), 108 (32, $[136 - CO]^+$); ^{1}H NMR (60 MHz, CD_3COCD_3) δ 6.9–7.9 (m, $CH(OH)_2$ and Ar); positive $FeCl_3$ test. Anal. Calcd for $C_8H_9NO_4$: C, 52.46; H, 4.95. Found: C, 52.24; H, 5.17.

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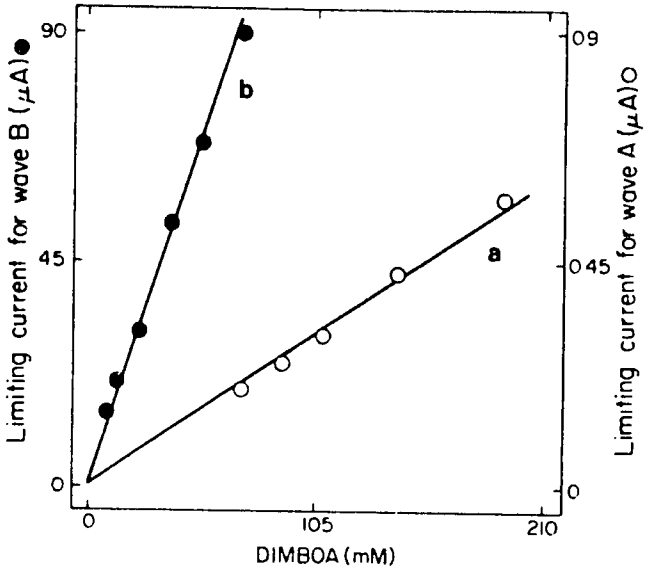


Figure 1. Variation of the limiting current with the concentration of DIMBOA for the reduction waves A (a) and B (b) in dimethylformamide.

Table I. Half-Wave Potentials for the Polarographic Reduction of DIMBOA and Model Compounds

compd[a]	solvent	$-E_{1/2}$/V vs. SCE wave A	wave B
DIMBOA	dimethylformamide	0.63	1.72
	dimethyl sulfoxide	0.62	1.95
	pyridine	0.65	1.73
PG	dimethylformamide	0.62	0.95
PGH	dimethylformamide	0.66	2.06

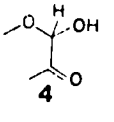
[a] Concentrations employed were: DIMBOA = 1.1 × 10^{-3} M; PG = 1.1 × 10^{-3} M; PGH = 1.4 × 10^{-3} M.

Results

Two reduction waves appeared in the polarographic reduction of solutions of DIMBOA in dimethylformamide. The limiting current for wave B was proportional to the square root of the height of the mercury reservoir, indicating diffusion control, and proportional to the concentration of DIMBOA (Figure 1b). Wave A was detected at DIMBOA concentrations greater than those used to detect wave B. Its limiting current was independent of the height of the mercury head, indicating kinetic control. The limiting current of wave A was over 100 times smaller than that of wave B and increased linearly with DIMBOA concentration (Figure 1a).

The polarographic behavior of solutions of DIMBOA in the other solvents employed was qualitatively similar.

The reduction of two model compounds was also studied: phenylglyoxal monohydrate (PG) and *N*-phenylglyoxylohydroxamic acid monohydrate (PGH). These compounds share with DIMBOA the structural feature depicted in 4. The polarograms of solutions of these com-



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pounds were similar to those of DIMBOA, showing a diffusion-controlled wave of type B and a wave with kinetic character of type A. These similarities suggest that electroactive groups are present in or are produced from the

Table II. Thermodynamic and Kinetic Constants for the Decomposition of DIMBOA at 28 °C[a]

solvent	$10^4 k_{obsd}$,[b] min^{-1}	$10^3 K$	k_2, min^{-1}
dimethylformamide	1.72	2.1	0.082
dimethyl sulfoxide	0.81	0.73	0.111
pyridine	0.52	0.27	0.194

[a] See eq 1 in the text. [b] Extrapoled to 28 °C from Arrhenius plots with data from ref 7.

partial structure 4. Half-wave potentials for the polarographic waves described are collected in Table I.

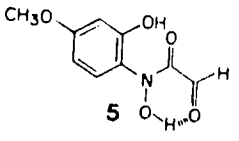
Discussion

Identification of Species Being Reduced. The polarographic reduction of DIMBOA solutions showed a kinetic wave A and a diffusional wave B. The half-wave potential of wave B was similar to that for the corresponding wave in PGH and fell within the range observed for the reduction of the hydroxamic acid moiety.[10] Hence, wave B may be attributed to the hydroxamic function in DIMBOA.

Waves showing kinetic control, such as wave A, have been reported in the polarographic reduction of sugars[11-14] and aldose oximes[15] and have been attributed to hemiacetal opening prior to electron transfer. On the other hand, similar waves have been reported in the polarographic reduction of PG[16] and of other aldehydes,[17] in aqueous solutions. They have been attributed to a dehydration process formally similar to hemiacetal opening (PG to 2). The waves of type A observed in the polarographic reduction of solutions of DIMBOA, PG, and PGH in nonaqueous solvents may thus be identified with compounds 1, 2, and 3, respectively.

Determination of Equilibrium Constant DIMBOA ⇌ 1. At sufficiently high concentrations of DIMBOA, small amounts of 1 could be detected directly. If the diffusion coefficients of cyclic and open-chain species, DIMBOA, and 1 are assumed to be equal, the equilibrium constants for hemiacetal opening can be determined as the ratio of the slopes of lines such as those shown in Figure 1 for dimethylformamide as solvent. Table II collects the values of such equilibrium constants in various solvents.

Studies of the hydroxyl stretching frequencies of DIMBOA in aprotic solvents have shown that the solvents interact with DIMBOA by sharing electron pairs with the hydroxyl groups at positions 2 and 4.[7] One of these interactions is expected to be absent in compound 1 since in it a strong intramolecular hydrogen bond would be formed (5). Hence, these solvents are expected to preferentially stabilize DIMBOA over compound 1. This



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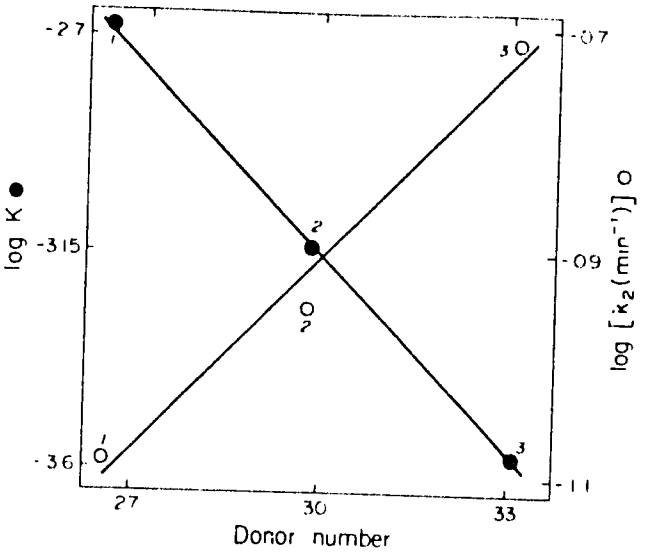
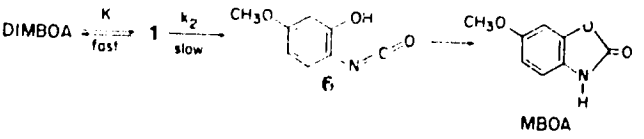


Figure 2. Effect of solvent donor number on the equilibrium constant for the process DIMBOA ⇌ 1 (○) and on the rate constant of formation of isocyanate 6 (Scheme I) (●). Solvents employed were dimethylformamide (1), dimethyl sulfoxide (2), and pyridine (3).

Scheme I



consideration is indeed reflected in the linear solvent energy relationship obtained with log K by using the donor number of the solvent, a measure of its ability to donate an electron pair (Figure 2).[18]

Mechanism of Decomposition of DIMBOA. DIMBOA decomposes to give 6-methoxybenzoxazolin-2-one (MBOA) as the main product both in protic[5,6,19] and aprotic[7] solvents. The comparable effects of basicity of aqueous solutions and donor ability of aprotic solvents on decomposition rate[5,20] and yield of MBOA[19] suggest that similar mechanisms are operating in both types of solvents. Evidence presented for the decomposition of DIMBOA in aprotic solvents gave support to the mechanism depicted in Scheme I.[7] In solvents of low donor number (lower than ca. 23), the opening of the hemiacetal is the rate-limiting step.[7] In solvents of higher donor number, such as dimethylformamide, dimethyl sulfoxide, and pyridine, the rate-limiting step is the formation of isocyanate 6. Under these conditions, the experimental rate constant for decomposition is equal to the product of the equilibrium constant for hemiacetal opening times the first-order rate constant for the formation of 6 (eq 1). The values for K

$$k_{obsd} = Kk_2 \quad (1)$$

determined in high donor number solvents allow the calculation of k_2 (Table II). The logarithms of these values correlated linearly with solvent donor number (Figure 2). Since the proposed formation of isocyanate 6 requires the nucleophilic attack of the hydroxamic hydroxyl group on the aldehyde function in 1, the rate enhancement observed in solvents of high donor number may be taken as further evidence for the mechanism of Scheme I.

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Registry No. 1, 103150-46-5; 6, 103150-45-4; DIMBOA, 69884-05-5; PG, 1075-06-5; PGH, 103150-44-3; MBOA, 532-91-2; PhN(OH)C(O)$CHCl_2$, 34282-44-5; PhNHOH, 100-65-2; $CHCl_2C$-(O)Cl, 79-36-7.

INTERACTION OF 2,4-DIHYDROXY-7-METHOXY-1,4-BENZOXAZIN-3-ONE (DIMBOA) WITH ALCOHOLIC SOLVENTS

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Abstract- Solubilities and IR spectra of DIMBOA in alcoholic solvents showed that the carbonyl group of DIMBOA is hydrogen-bonded to the solvent. Activation parameters of DIMBOA in the same solvents suggested that the decomposition of DIMBOA occurs through an isocyanate, in analogy with the reaction in aqueous and aprotic solvents.

Extracts of Gramineae such as wheat and maize contain hydroxamic acids, such as DIMBOA, which are involved in the resistance of the plants to pests and pathogens.[1,2] The study of the chemistry of DIMBOA has provided a basis for rationalizing its biological properties at the molecular level.[3] The stability of DIMBOA has been described in aqueous[4,5] and aprotic solvents.[6] We herein describe the behavior of DIMBOA in alcoholic solvents.

Solubilities of DIMBOA were determined by adding $FeCl_3$ reagent to the supernatant of saturated solutions of DIMBOA and measuring the absorbance at 590 nm of the blue complex formed. Rates of decomposition of DIMBOA were followed by taking aliquots of the reaction mixture after different time intervals and analyzing them with $FeCl_3$ reagent.

Solubilities of DIMBOA in different alcohols generated a linear solvation energy relationship (Fig. 1) using acceptor number (AN) as solvent parameter, a measure of the ability of the solvent to accept an electron pair.[7,8] Additionally, a 2-parameter correlation using AN and DN (donor number), a measure of the ability of the solvent to donate an electron pair,[7,8] produced a ratio of coefficients for AN and DN equal to 25, further supporting that electron pair accepting properties of the solvent are of overwhelming importance in its interaction with DIMBOA.[9]

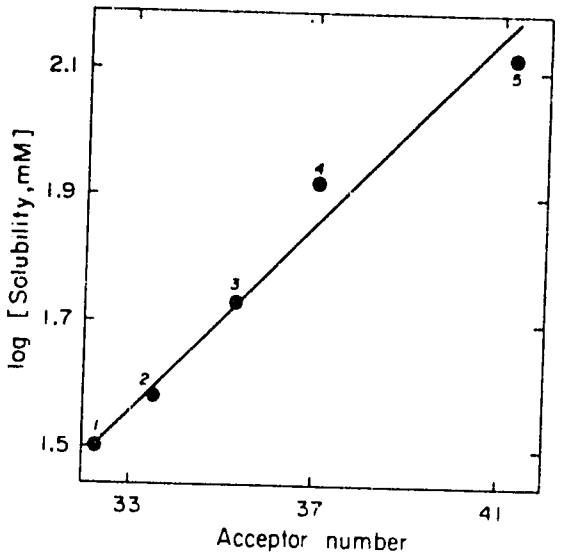


Fig. 1. Effect of the acceptor number of alcoholic solvent on the solubility of DIMBOA at 284 K. Solvents employed were: methanol (1), ethanol (2), 1-propanol (3), 2-propanol (4) and 1-butanol (5). Correlation coefficient = 0.986.

These results point to the interaction of electrophilic sites in the solvent with nucleophilic sites in DIMBOA. The former can be assumed to be the hydroxyl proton in the solvent; the latter may be identified with the carbonyl group of DIMBOA. Confirmation of this proposition was obtained from the effect of solvent on the carbonyl stretching frequency of DIMBOA. Table 1 shows that as AN increased, the carbonyl band broadened and shifted towards the region of increased single bond character, indicating hydrogen bonding of the carbonyl group to the solvent.

Table 1. Stretching frequencies and band widths of the carbonyl group in DIMBOA dissolved in alcohols

Solvent	$\nu_{C=O}$ (cm^{-1})	$\Delta\nu$ (cm^{-1})	AN[a]
Methanol	1672	very broad	41.3
Ethanol	1684	57	37.1
1-Propanol	1689	48	35.3
2-Propanol	1687	48	33.5
1-Butanol	1687	47	32.2
t-Butanol	1689	40	27.7

[a]see note 8.

The kinetics of decomposition were first order with respect to DIMBOA. Free energies of activation produced a linear solvation energy relationship with AN (Fig. 2). In addition, an isokinetic relationship (correlation coefficient = 0.998) was generated with a value of β (363 K) falling within the range expected for solvent effects on reaction rates.[10]

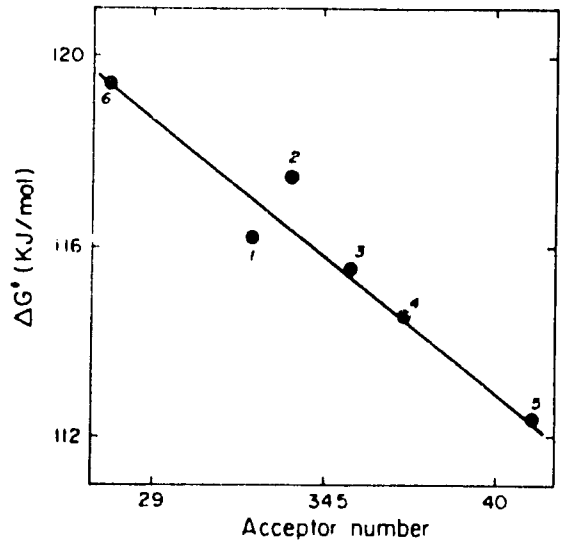
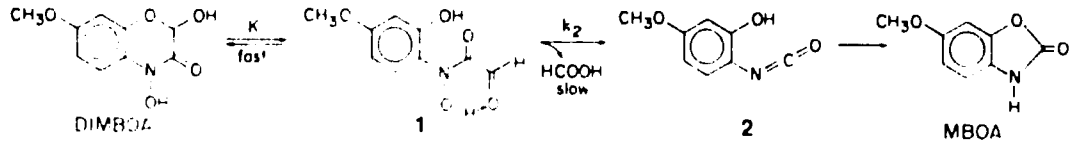


Fig. 2. Effect of the acceptor number of the alcoholic solvent on the free energy of activation of DIMBOA at 326 K. Solvents employed were the same as in Fig. 1 and t-butanol (6). Correlation coefficient = -0.980.

The main products from the reaction were 6-methoxybenzoxazolin-2-one (MBOA) and formic acid. The following mechanism incorporates the features described:



The observed rate constant is the product of the equilibrium constant for hemiacetal opening (K) times the rate constant for isocyanate formation (k_2). Intermediate 1 has a carbonyl group engaged in intramolecular hydrogen bonding and a carbonyl group relatively free to interact with the solvent. Thus 1 does not differ substantially from DIMBOA in capability of interaction with solvent

in the manner previously described. Hence, electron pair accepting properties of the solvent are not expected to show a substantial influence on the value of K. On the other hand, during formation of the transition state leading to isocyanate 2, the intramolecular hydrogen bond in 1 must be broken to allow attack by the hydroxamic hydroxyl group on the aldehydic carbon. This process is expected to be facilitated by accepting solvents which not only compete with the hydroxamic hydroxyl group for interaction with the carbonyl group but also polarize it increasing its receptivity towards nucleophilic attack. This is borne out by the free solvation energy relationship of Fig. 2.

A mechanism similar to that presented has been postulated for the decomposition of DIMBOA in aqueous[4] and aprotic[6,11] solvents.

ACKNOWLEDGEMENTS

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Chapter 12

Resistance of Cereal Crops to Aphids: Role of Allelochemicals

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Aphids cause important economic losses in cereals. Thus, development of resistant varieties is desirable. This paper describes the possible role of various natural chemical compounds in the resistance of barley, wheat, triticale and maize to the aphids *Schizaphis graminum*, *Rhopalosiphum padi* and *Metopolophium dirhodum*. Resistance of barley to aphids may be affected by the concentration of the alkaloid gramine. Conversely, increased susceptibility of barley seedlings grown under water stress is partially caused by accumulation of glycine-betaine in their leaves. Resistance of wheat, triticale and maize to these insects is mainly determined by the presence of hydroxamic acids.

Resistance of plants to herbivorous animals is determined in many cases by the presence in plant tissues of secondary metabolites that may cause feeding deterrence or toxocity to the animal. Insects cause damage to cereals, decreasing grain production. Development of varieties resistant to insects is then desirable for improving cereal productivity.

Resistance of barley to aphids has been reported to be influenced by the presence of several phenolic and flavonoid compounds (1). Gramine and related indole alkaloids cause toxicity in ruminants feeding on various Gramineae and Leguminosae (2,3). These alkaloids have also been reported to cause toxicity and feeding deterrence to aphids (4,5). Since these compounds also decrease palatability of fodder plants, it has been proposed to reduce the alkaloid content of various Gramineae by plant breeding (6). Resistance of Sorghum to the greenbug *Schizaphis graminum* appears to be affected by the degree of methylation of the intercellular pectin (7). Sorghum (8), barley (9), and other cereals have cyanogenic glucosides that may be important in plant protection. Dhurrin was identified as a feeding deterrent towards the greenbug (10). Dhurrin is located entirely in epidermal cells of Sorghum leaves (11). Hydroxamic acids may be important in determining the resistance of maize to the European corn borer *Ostrinia nubilalis* (12) and of wheat and maize to aphids (13 - 15). These compounds are found more concentrated in the vascular tissues than in other parts of the leaf (16).

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In this paper we summarize our work on the role of indole alkaloids and hydroxamic acids on the resistance of cereals to aphids. In addition, we describe the effects of water stress on susceptibility of barley to aphids.

Results

Susceptibility to aphids and gramine and hydroxamic acid content. Seedlings of several species of cereals were infested with aphids. At this time, the content of gramine and hydroxamic acids in the leaves was measured. Six days later aphid population growth rate was measured. Gramine was found only in barley while hydroxamic acids were present in wheat, triticale, and maize (Table I). Correlations were observed between resistance of barley to aphids and gramine content. Correlation coefficients between gramine content and population growth rate of *Rhopalosiphum padi* and *Metopolophium dirhodum* were -0.99, and -0.96, and -0.99, respectively. *R. padi* was more affected than *S. graminum* and *M. dirhodum* by the gramine content of leaves. Similarly, hydroxamic acid content of leaves of wheat, triticale and maize correlated with resistance to *S. graminum* and *M. dirhodum*.

Distribution of compounds in barley and wheat tissues. Tissues of barley and wheat leaves were mechanically separated under the microscope. It was observed that in barley gramine was more concentrated in the epidermis than in the entire leaf (Table II). Hydroxamic acids in wheat were absent in epidermic tissues and were more concentrated in the vascular tissues than in the entire leaf. Neither compound was detected in xylem exudates nor in guttation drops.

Biological activity of gramine and hydroxamic acids. Gramine decreased survival, feeding, and reproduction of aphids in artificial diets (Table III) at a concentration similar to those found in leaves of several barley cultivars. DIMBOA, 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (the main hydroxamic acid from wheat and maize), and its glucoside also showed deleterious effects on the aphids at concentration levels found in leaves of wheat and maize.

Effect of water stress on susceptibility of barley to aphids. Barley seedlings were subjected to water stress. These plants accumulated among other compounds proline and glycine-betaine (Table IV). Also, the more stressed plants were the most susceptible to aphids. The cultivar used in this experiment lacked gramine. However, when a gramine-containing cultivar was used gramine concentration was not affected by water stress.

To test if some stress compounds had beneficial effects on aphids proline, choline and glycine-betaine were incorporated into artificial diets. Proline and choline appeared to decrease survival of aphids, while glycine-betaine did not (Table V). Moreover, glycine-betaine caused a drastic increase in aphid reproduction. Thus, the increased susceptibility to aphids of water stressed plants may be partially due to the higher content of glycine-betaine.

Table 1. Gramine and Hydroxamic Acid Content and Susceptibility of Various Cereals to Aphids

	Compounds (mmoles/kg fr wt)		Population Growth Rate (day^{-1})		
	Gramine	Hydroxamic Acid	Sg	Rp	Md
Hordeum distichum+					
cv F. Union	ND	ND	0.38	0.32	0.43
Brea "S" Celaya	1.2	ND	0.35	0.24	0.40
79 AN MX	1.7	ND	0.33	0.15	0.38
Abyssinian 5	2.9	ND	0.30	0.12	0.34
Triticum aestivum+					
cv Sonka	ND	0.6	0.44	-	-
Likay	ND	0.9	0.43	-	-
Cajeme	ND	1.5	0.24	-	-
Naofen	ND	1.5	0.24	-	-
T. durum+					
cv SNA-1	ND	1.8	0.21	-	-
T. aestivum++					
cv Huenufen	ND	0.5	-	-	0.35
Nacfer	ND	1.2	-	-	0.31
T. durum++					
cv SNA-3	ND	3.1	-	-	0.23
Triticale—	ND	4.5	-	-	0.14
Zea mays—					
cv T115 L21	ND	7.3	-	-	all dead

The infestation was carried out on 10-day-old (+) or 7-day-old (++) seedlings in greenhouse-grown plants. After six days population growth rate was determined (growth rate = ln (Nf/Ni)/Δt). Sg, *Sitobion graminum*; Rp, *Rhopalosiphum padi*; Md, *Metopolophium dirhodum*. ND, not detected.

Table II. Distribution of Gramine and Hydroxamic Acids in Barley and Wheat Tissues

Leaf Part	Compound (mmoles/kg fr wt) Gramine	Hydroxamic Acids
Complete leaf	0.28	4.2
Veins	ND	7.0
Guttation drops	ND	ND
Xylem exudate	ND	ND
Epidermis	0.72	ND

Gramine was determined in barley (cv XB1-T-1031) and hydroxamic acids in wheat (cv SNA-3) tissues. Plants were grown for 10 days in a greenhouse with a day-time temperature of 25°C and a night-time temperature of 16°C. Tissues were mechanically separated under a microscope.

Table III. Effects of Gramine, DIMBOA and DIMBOA-Glucoside on *Schizaphis graminum* feeding on artificial diets

Compound	Survival (%)	Aphids feeding (%)	Reproductive Index
None	100	60	4.0
Gramine	10	25	1.8
DIMBOA	15	0	3.0
DIMBOA-Glucoside	50	-	3.4

Survival after 24 h and the number of aphids feeding were determined at 4 mM concentration in the diet. Reproductive index (number of nymphs average number of adults) was determined at 0.15 mM concentration in the diet. Values are the average of three samples of ten aphids each. For reproduction studies five samples were used. Standard errors were always less than 10%. DIMBOA: 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one.

Table IV. Effect of Water Stress in Barley Plants on Proline and Glycine-Betaine Content and Susceptibility of Barley to *Schizaphis graminum*

Water Potential (-bars)	Proline (mmoles/kg dry weight)	Betaine	Population Growth Rate (day^{-1})
6.3	5	57	0.36
10.6	11	79	0.39
15.5	25	126	0.43

Barley seeds (cv F. Union) were germinated at 25°C and after 6 days were subjected to water stress. Four days later, plants were analyzed for proline and betaine content and the water potential of the leaves was measured. At this time, plants were infested with aphids and the insects were counted six days later.

Table V. Effects of Proline, Choline and Glycine-Betaine on *Schizaphis graminum* Feeding on Artificial Diets

Compound	Survival (%)	Aphids Feeding (%)	Reproductive Index
None	93 ± 3	90 ± 4	2.45 ± 0.10
Proline	96 ± 6	57 ± 3	2.13 ± 0.13
Choline	72 ± 5	92 ± 3	2.00 ± 0.29
Glycine-betaine	98 ± 3	74 ± 5	3.63 ± 0.11

Biological assays were performed with [illegible] mM compound in the diet. The reproductive index of aphids fed with 11 mM of glycine-betaine was [illegible] after [illegible] h of feeding. Values shown are the average ± standard error of three samples of ten aphids each. Reproduction studies were performed with five samples.

Discussion

Correlations between content of various compounds in cereals and population growth rate of aphids on the plants were found. In addition, these compounds cause deleterious effects on aphids on artificial diets at concentrations similar to those found in plants. Thus, it is possible that gramine and hydroxamic acids play a role in protecting barley and wheat, respectively, against aphids.

S. graminum feeds preferentially from phloem (17). Hydroxamic acids are more concentrated in the vascular bundles of wheat leaves than in other tissues. Moreover, these compounds are not present in

xylem exudates. Although the exact location of hydroxamic acids within the veins is unknown, it is likely that these compounds protect phloem against aphid feeding. Gramine was found preferentially in the epidermis of barley and was not detected in the veins. Thus, gramine is not found in the main feeding site (phloem) of the greenbug. Nonetheless, the feeding deterrent properties of gramine may be relevant to plant protection.

A variety of compounds may accumulate in plants under water stress (18, 19). One of these compounds, glycine-betaine, increased reproduction rates of aphids in artificial diets. It is likely that the observed increased susceptibility of barley to aphids may be due to glycine-betaine accumulation in barley leaves. It is possible that cultivars that accumulate preferentially proline and other amino acids instead of glycine-betaine under stress conditions may be more resistant to aphids.

Experimental

Analyses of compounds. Plant tissues were homogenized in water, adjusted to pH 3, and centrifuged at 3500 g for 15 min. The supernatant fluid was extracted with diethyl ether (2:1 v/v, 2x) and the extract evaporated to dryness. This extract was used for quantitation of hydroxamic acids as previously described (14).

The samples for gramine analyses were frozen and macerated with 20 ml of MeOH: NH_4OH (100:1 v/v). The extract was filtered through glass wool. Solvent and endogenous water were evaporated to dryness. The chlorophyllous residue was dissolved in 5 ml 0.1 M HCl, and the solution filtered (Whatman N°1 paper). The aqueous filtrate was adjusted to pH 9 with concentrated NH_4OH and shaken twice with chloroform (1:2 v/v). The organic phase was evaporated to dryness. Gramine was quantified in these extracts by using Ehmann's reagent for indoles (20), as described previously (5).

Proline was quantified by the method of Bates *et al.* (21). Glycine-betaine was quantified by the method of Grieve and Grattan (22).

Feeding assays. Assays were performed with diets placed between two layers of Parafilm M (23). The diet was as described (14). When young aphids were used (survival and feeding deterrence assays) they were 3rd and 4th-instar nymphs.

Water stress treatment. Four-day-old seedlings were kept at 28°C (without irrigation for 48 h). Three groups of plants were then watered daily with different amounts of water for six days. Water potential of leaves was measured at this stage (25).

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GLYCINE-BETAINE ACCUMULATION INFLUENCES SUSCEPTIBILITY OF WATER-STRESSED BARLEY TO THE APHID *SCHIZAPHIS GRAMINUM*

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(*Revised received* 6 *June* 1986)

Key Word Index – *Hordeum*, Gramineae, barley; *Schizaphis graminum*, greenbug; aphids; insect resistance; water stress; betaine, proline

Abstract – Water deficit increased susceptibility of barley to the aphid *Schizaphis graminum*. Proline and glycine betaine accumulated in the stressed plants. These compounds were incorporated into artificial diets to test their effects on aphids. Survival of *S. graminum* was not affected by proline and glycine-betaine. In addition, glycine-betaine increased reproduction of the greenbug at concentrations similar to those found in stressed barley plants. When glycine betaine was added to detached shoots of barley, population growth rate of *S. graminum* increased in that plant material kept in the betaine solutions. It is suggested that glycine-betaine accumulation may be responsible for the increased susceptibility of water-stressed barley to the greenbug.

INTRODUCTION

Barley leaves accumulate proline and glycine betaine under water stress [1]. The grain yields of barley cultivars that accumulate proline as a response to water stress are not decreased over a wide range of environments [2]. However, proline accumulation does not serve as an index of drought resistance [3]. Although a precise role has yet to be assigned to glycine-betaine, this compound may function as an organic cytoplasmic osmoticum [4]. The finding that glycine betaine is located in the cytoplasm supports this hypothesis [5]. Moreover, proline and glycine betaine protect several enzymes against *in vitro* heat inactivation [6]. It has also been reported that proline concentration increases in virus infested leaves [7]. Hagiuni [8] suggested that plants with a higher content of proline are more susceptible to insect predators but it was later shown that proline content does not influence susceptibility of barley [9]. Betaine and choline concentration increased following rust infection in susceptible but not in resistant wheat varieties [10]. However, a direct correlation between the susceptibility and the concentration of these compounds has not been demonstrated. We report here that accumulation of glycine-betaine in water-stressed barley may influence the susceptibility of seedlings to the aphid *Schizaphis graminum* (Rondani).

RESULTS AND DISCUSSION

Water stress and susceptibility of barley

Barley seedlings subjected to water stress were infested with adult apterous aphids. Aphid population growth rate increased in the stressed plants (Fig. 1a). These plants also accumulated glycine-betaine and proline (Fig. 1b). To test if these compounds were responsible for the increased susceptibility, detached shoots and leaves were put in solutions containing proline, choline and glycine-betaine. After two days each group was infested with adult aphids and the population growth rate was determined six days later (Fig. 2). Population growth rate increased in plants kept in the glycine-betaine solutions. Proline and choline produce a small decrease in the population growth rate.

Aphid feeding experiments

Proline, choline and glycine-betaine were incorporated into artificial diets to test their effects on the greenbug. Choline decreased survival of aphids, while proline and glycine betaine did not (Table 1). Moreover, glycine betaine increased reproduction of the greenbug at concentrations similar to those found in plants (Table 1 and Fig. 3).

Our results have shown correlations between natural accumulation of glycine betaine and plant susceptibility. Also, a similar correlation was found when glycine betaine concentrations of the leaves were artificially increased by putting the plants in glycine-betaine solutions. Moreover this compound increased the reproduction of aphids reared with artificial diets. Thus, it is suggested that glycine-betaine accumulation may be a factor responsible for increasing the susceptibility of water stressed barley to the aphid *S. graminum*. Whether other compounds involved in water stress responses also increase susceptibility to aphids remains to be determined. Since proline and choline did not show beneficial effects on aphids reared on artificial diets, it is possible that cultivars which preferentially accumulate these compounds are more resistant to aphids than those that accumulate glycine-betaine.

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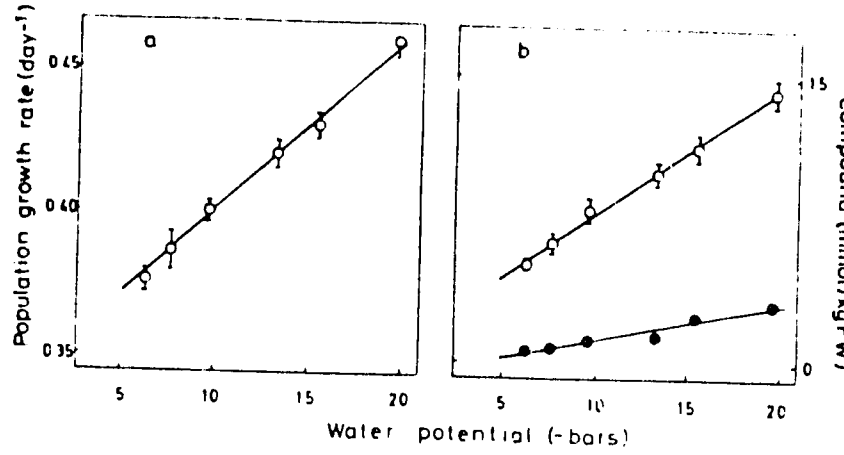

Fig. 1. Effect of water stress on the susceptibility of barley plants and proline and glycine betaine content. Barley seeds cv. F. Union, were grown in soil a 16 hr photoperiod in a growth chamber at 25 ± 2°. Four-day-old seedlings were kept without irrigation for 48 hr. Five groups of plants were then irrigated daily with different amounts of water for six days. Leaf water potentials were measured in ten-day-old seedlings using a pressure chamber. Replicate groups were infested with adult apterous aphids. Insect population growth rate was determined six days after (Fig. 1a). Each value is the mean of three samples of ten plants each. Vertical bars are 1 s.e. Population growth rate = $\ln(N_f/N_0)/\Delta t$. Proline (●) and betaine (○) (Fig. 1b) were quantified as described in the experimental section.

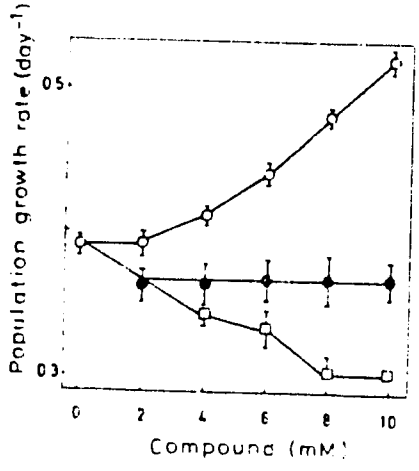

Fig. 2. Effect of proline, choline and glycine betaine incorporated in detached shoots of barley on the susceptibility to aphids. Detached shoots of eight-day-old barley seedlings cv. F. Union, were put in solutions containing proline, choline and glycine betaine. After two days each group was infested with two adult apterous aphids. Population growth rate was determined six days after as in Fig. 1. Each value is the mean of three samples of five plants each. Vertical bars are 1 s.e. Solutions contained 10^{-4} M kinetin to delay senescence of leaves. At this concentration kinetin did not have detectable effects on the greenbug. Glycine betaine (○), proline (●), choline (□).

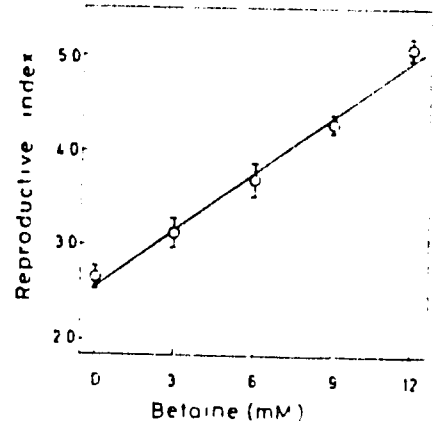

Fig. 3. Effect of glycine betaine on reproduction of *S. graminum* reared on artificial diets. Adult aphids were fed with artificial diets with glycine betaine added. The reproductive index was measured after 72 hr. Each value is the mean of three samples of ten aphids. Vertical bars are 1 s.e. The reproductive index was the amount of nymphs born divided by the average number of adults. All values were significantly different at $P < 0.05$ by t test.

EXPERIMENTAL

Analyses of compounds. Proline was quantified by the method of Bates *et al.* [11]. Plant material (0.5 g) was homogenized in 10 ml of 3% aqueous sulphosalicylic acid and then filtered through Whatman No. 1 filter paper. Two ml of this filtrate were incubated with 2 ml acid-ninhydrin and 2 ml HOAc at 100° for 30 min and the reaction terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene and the absorbance was measured at 520 nm using toluene for a blank (ε_{520} = 11 511).

Glycine betaine was quantified by a method previously described [12]. Dried plant material (0.5 g) was shaken with 20 ml of deionized H_2O for 24 hr at 25°. The extracts were diluted 1:1 with 2 N H_2SO_4. Aliquots of 0.5 ml were cooled in ice water for 1 hr and then 0.2 ml of cold KI-I_2 reagent was added. The tubes were stored at 0–4° for 16 hr and then centrifuged at 5,000 *g* for 30 min at 0°. The supernant was discarded. The periodide crystals were dissolved in 5 ml 1,2-dichloroethane. After 2.5 hr the absorbance was measured at 365 nm (ε_{365} = 2244).

Water stress treatment. Five groups of four-day-old seedlings were watered daily with different amounts of water for six days. Water potential of leaves was measured at this stage [13]. Then, each group of plants was infested with adult apterous aphids of *S. graminum*. The number of aphids on the leaves was measured six

Table 1. Effects of proline, choline and glycine betaine on *Schizaphis graminum* feeding on artificial diets

Compound	Survival (%)	Amount of diet ingested (mg/10 aphids)	Reproductive index (nymphs/adult)
None	93 ± 3	0.9	2.5 ± 0.3
Proline	96 ± 6	0.9	2.3 ± 0.1
Choline chloride	72 ± 5*	0.7*	2.2 ± 0.2
Glycine-betaine	98 ± 3	0.9	3.3 ± 0.2*

Biological assays were performed with holidic diets placed between two layers of parafilm M. The diet was as described in the Experimental with 6 mM of each compound added. Survival of aphids was measured after 48 hr of feeding. The amounts of diet ingested were measured by weighing the parafilm sachets after 7 hr. The reproductive index was measured after 72 hr. All assays were performed with adult apterous aphids. Values shown are the mean of three samples of ten aphids each ± 1 s.e. Reproductive index is the amount of nymphs born divided by the average number of adults. (*) = significant differences from control values at $P < 0.05$ by t test.

days after and population growth rate was calculated (population growth rate = $\ln(N_f/N_i)/\Delta t$).

Feeding assays. Assays were performed with diets placed between two layers of Parafilm M [14]. The diet was a solution containing 35% sucrose, amino acids and mineral salts adjusted to pH 6.0. Three samples of ten aphids each were used for each treatment.

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INHIBITION OF ENERGY METABOLISM BY BENZOXAZOLIN-2-ONE

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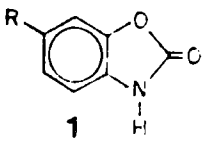
Abstract—1. The effects of the title compound (BOA) on energy-linked reactions in mitochondria were studied

2. BOA inhibited electron transfer between the flavin and ubiquinone in Complex I, and ATP synthesis at the F_1 moiety of the ATPase complex

3. These results are discussed in relation to the toxicity of BOA towards a wide range of aerobic organisms

INTRODUCTION

Benzoxazolin-2-ones such as compounds 1 (Scheme 1) are decomposition products of hydroxamic acid glucosides naturally occurring in Gramineae such as maize, wheat and rye, their production starting when the plant tissue is injured (Hofman and Hofmanova, 1971).



BOA R = H
MBOA R = CH_3O

1

The possible role of benzoxazolin-2-ones as host plant resistance factors towards various pests and pathogens has been investigated. Benzoxazolin-2-one (BOA) and its 6-methoxy derivative (MBOA) produce deletereous effects on various organisms

MBOA is toxic to the cereal aphid *Metopolophium dirhodum* (I_{50} = 7 mM) (Argandoña *et al.*, 1980). Benzoxazolin-2-ones inhibit growth of larvae of the European corn borer, *Ostrinia nubilalis* (I_{50} = 2.5 mM) (Beck and Smissman, 1961) and of other insects such as *Bombyx mori* (Kubo and Kamikawa, 1983) and *Blatella germanica* and *Prodenia eridana* (Beck and Stauffer, 1957).

The growth of *Fusarium nivale*, a fungus pathogenic to rye, is completely inhibited by 3 mM BOA or MBOA (Wahlroos and Virtanen, 1958). Other plant pathogenic fungi such as *Fusarium moniliforme*, *Gibberella zeae*, *Pyrenochaeta terrestris*, *Diplodia zeae* (Whitney and Mortimore, 1959), *Sclerotinia trifoliorum* (Virtanen *et al.*, 1957) and *Penicillum chrysogenum* (Beck and Smissman, 1961) are also inhibited at concentrations ranging from 1 to 6 mM.

Plant pathogenic bacteria such as *Xanthomonas stewardii* are inhibited by MBOA at 4 mM (Whitney and Mortimore, 1961). In addition, both BOA and MBOA inhibit growth of *Staphylococcus aureus*, *Pseudomonas fluorescens* and *Escherichia coli* at concentrations higher than 5 mM (Virtanen *et al.*, 1957).

The toxicity of benzoxazolin-2-ones towards such a wide range of aerobic organisms suggests that the energy metabolism might be a possible target for their action. We report herein the effects of BOA on mitochondrial electron transport and ATP synthesis.

MATERIALS AND METHODS

Phosphorylating submitochondrial Mg^{2+}-ATP particles (SMP) were prepared from heavy bovine heart mitochondria (Smith, 1967) as described (Low and Vallin, 1963). Beef-heart F_1 was prepared according to Knowles and Penefsky (1972). It was stored at liquid nitrogen temperature and processed before used as described (Roveri and Calcaterra, 1985)

Electron transport

Electron transport in SMP (0.3 mg protein) was determined following oxygen consumption with a Clark electrode connected to a Gilson oxygraph in a reaction medium containing 250 mM sucrose, 50 mM Tris-HCl (pH 7.5), 50 mM ethanol, 125 I.U./ml of yeast alcohol dehydrogenase (EC 1.1.1.1) and NADH. Rates reported correspond to the slopes of linear traces drawn by the oxygraph

Reduction of flavoproteins and cytochrome b in SMP

The reduction kinetics were followed by determining the absorbance changes in an Aminco DW-2a spectrophotometer (Dual wavelength mode) at 475 minus 510 nm (flavoproteins) or 563 minus 575 nm (cytochrome *b*). The reaction was carried out at 2°C in a medium containing 50 mM potassium phosphate (pH 7.5) and SMP (2.5 mg protein/ml)

ATP synthesis

The reaction was measured in a medium containing (final conc.) 180 mM sucrose, 1 mM $MgCl_2$, 10 mM succinate, 0.5 mM EDTA, 3 μM rotenone, 50 mM glucose, 2 mM ADP, 15 I.U. of yeast hexokinase (EC 2.7.1.1) and 50 mM Tris-HCl pH 7.4. SMP (0.25 mg protein) were added to this medium and incubated for 5 min. ATP synthesis was started by adding 5 μmoles P_i, 2×10^6 cpm carrier free $^{32}P_i$ and 3 μmoles $MgCl_2$. The final vol. was 1 ml. The preincubation and the reaction were carried out under aerobiosis obtained with a giratory waterbath shaker. After 5 min the reaction

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was stopped and inorganic phosphate was quantitatively precipitated essentially as described by Sugino and Miyoshi (1964). After separating the ppt. by centrifuging for 10 min at 3000 rpm, aliquots were withdrawn and analyzed for (^{32}P)glucose-6-phosphate by Cerenkov counting in a Beckman 8100 liquid scintillation counter.

P_i-ATP exchange reaction

SMP (1 mg protein) were added to 1 ml of a reaction medium containing 180 mM sucrose, 10 mM ATP, 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5), 10 mM P_i and 2×10^6 cpm of carrier free $^{32}P_i$. After 5 min the reaction was stopped by adding Sugino and Miyoshi's reagent (1964) and after centrifuging at 3000 rpm for 10 min the supernatant was analyzed for (γ – ^{32}P)ATP by Cerenkov counting.

ATPase activity of SMP and of F_1

ATPase activity was determined spectrophotometrically as described (Pullman et al., 1960) in a reaction medium (2 ml) containing 100 mM sucrose, 1 mM $MgCl_2$, 4 mM phosphoenolpyruvate, 0.125 mM NADH, 60 I.U. pyruvate kinase (EC 2.7.1.40), 50 I.U. lactate dehydrogenase (EC 1.1.1.27), 40 mM Tris-HCl (pH 8.0) and Mg-ATP. The reaction was started by adding F_1 (3 μg protein) or SMP (0.1 mg protein) to the reaction medium. In the latter case, the medium also contained 3 μM rotenone.

General

Protein determinations were carried out according to Lowry et al. (1951).

BOA (Aldrich Chem. Co.) was recrystallized twice before use and dissolved in dimethylsulfoxide prior to its addition to the reaction medium. The solvent (less than 2%) had no effect on any of the biochemical reactions studied.

Measurements were carried out at 30°C, except when indicated.

Rates informed are the average of duplicate experiments which agreed within 10%.

RESULTS AND DISCUSSION

BOA completely inhibited electron transport from NADH to oxygen in SMP (Fig. 1). This effect can be located at the level of the mitochondrial respiratory chain since similar results were obtained in the presence of uncouplers (data not shown). In addition, since succinate oxidation was not inhibited it can be suggested that BOA inhibits electron transport from NADH to ubiquinone (complex I of the respiratory chain).

A more precise location was accomplished by following the absorbance changes produced by addition of NADH or NADH plus succinate, in the flavoprotein spectral region (475 minus 510 nm): (i) NADH-dependent absorbance decrease in SMP was partially inhibited by BOA. Maximal inhibition (about 50%) was obtained with 10 mM BOA (Fig. 2B), a concentration which almost completely inhibited cytochrome *b* reduction (Fig. 2A); (ii) subsequent addition of succinate to NADH-reduced BOA-inhibited SMP (Fig. 2B) increased the absorbance change to a level similar to that observed in the absence of BOA; (iii) similar results were obtained with rotenone (Fig. 2B), a known inhibitor that blocks electron transport from FMN to ubiquinone in complex I, and (iv) the addition of rotenone to NADH-reduced BOA-inhibited SMP did not produce any extra inhibition (Fig. 2B).

Half of the absorbance change at 475 minus 510 nm can be attributed to reduction of the FMN moiety of NADH dehydrogenase (Hatefi, 1985), which is the first acceptor of electrons from NADH. Therefore, it can be concluded that FMN is in the substrate side of the BOA-sensitive site. The nature of the electron transport chain components responsible for the other half of the absorbance change and their relative contribution has not been clearly established yet. Nevertheless, it has been reported (Boveris and Stoppani, 1970) that the major contribution to the signal corresponds to flavoprotein reduction (FMN of NADH dehydrogenase and FAD of succinate dehydrogenase). In addition, under our experimental conditions, NADH was able to completely reduce succinate, since the same extent of absorbance decrease was obtained in the presence of either NADH or NADH plus succinate (data not shown). Therefore, most of the BOA-sensitive NADH-dependent absorbance decrease could be attributed to FAD and ubiquinone reduction. Hence, FAD and ubiquinone would be located on the oxygen side of the BOA-sensitive site. Since BOA and rotenone exerted similar effects and the addition of rotenone to BOA-inhibited SMP did not produce any extra inhibition, it can finally be concluded that BOA exerts its action at or very near to the rotenone-sensitive site.

ATP synthesis driven by succinate oxidation was also inhibited by BOA (50% inhibition was obtained with 2 mM BOA, Fig. 1). This effect cannot be attributed to inhibition of electron transport since, as stated above, succinate oxidation was insensitive to BOA. This result together with the inhibition of P_i-ATP exchange reaction and ATPase activity (Fig. 1) in SMP indicated that a second probable site of action of BOA is the ATPase complex. Furthermore, since ATPase activity of the soluble mitochondrial ATPase (F_1) was also inhibited by BOA (Fig. 1), the

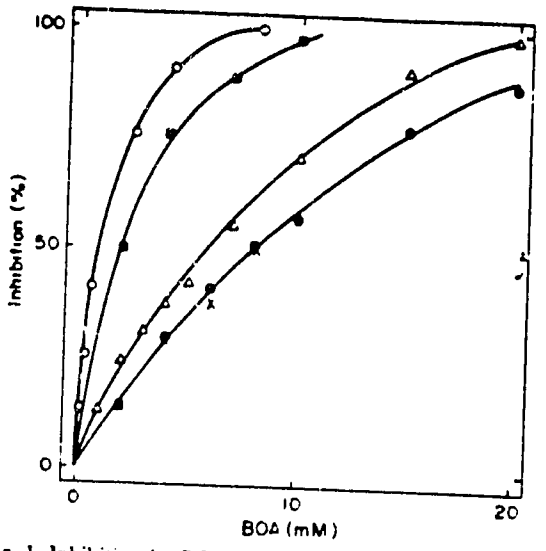

Fig. 1. Inhibition by BOA of several mitochondrial energy-linked reactions. The reactions measured (particular conditions in parenthesis) were: P_i-ATP exchange reaction (○), ATP synthesis driven by succinate oxidation (■), NADH oxidation (△, 0.3 mM NADH), ATP hydrolysis catalyzed by SMP (●, 1 mM Mg-ATP) or by F_1 (×; 0.07 mM Mg-ATP). Activities for controls were: 102, 315 nmol/min mg protein, 450 nat/min mg protein, 1.25 and 25 μmol/min mg protein, respectively.

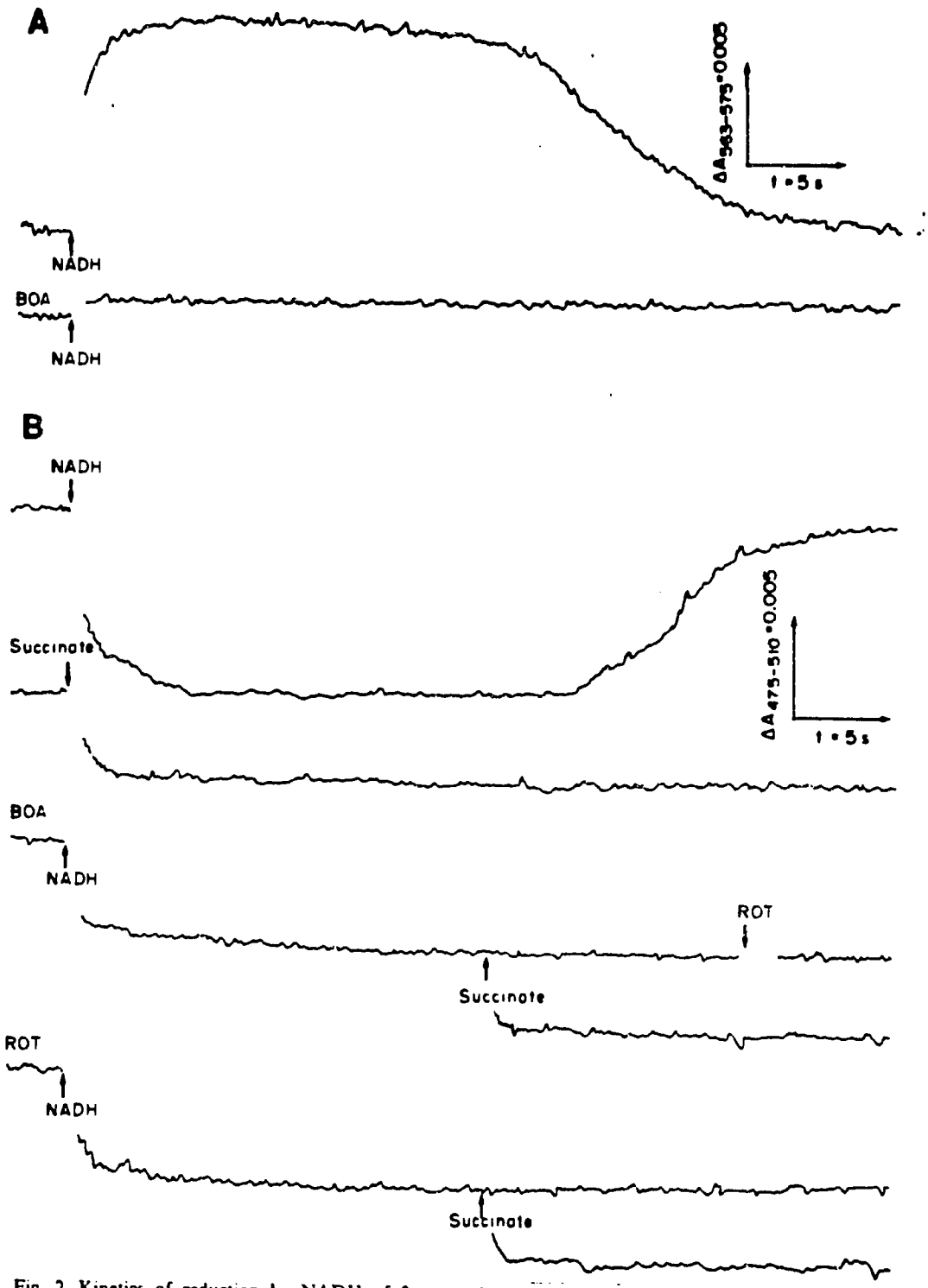

Fig. 2. Kinetics of reduction by NADH of flavin and cytochrome *b* in SMP. The time course of cytochrome *b* (A) and flavin (B) reduction and reoxidation was determined as described under Materials and Methods. Additions were NADH (0.45 mM), ROT (3 μM rotenone), BOA (10 mM) and succinate (10 mM).

likely site of action of BOA is at the F_1 moiety of the ATPase complex. BOA behaved as an uncompetitive inhibitor with respect to ATP for the ATPase activity of isolated F_1 (K_i = 3 mM, Fig. 3) and as a non-competitive inhibitor with respect to ATP in SMP (K_i = 8 mM, Fig. 4). Hence it can be concluded that the binding of BOA does not occur at the active site of the ATPase complex. The differences between the inhibitions by BOA of the ATPase activities of isolated F_1 and of SMP, could reflect different conformational states or different mechanisms of soluble and membrane bound F_1, *viz.* in isolated F_1 only the kinetically competent F_1–ATP complex would bind BOA, whereas in membrane-bound F_1, both F_1 and the F_1–ATP complex would be equally able to bind BOA.

The above discussed effects of BOA resemble somehow the effects described for hormonal steroids

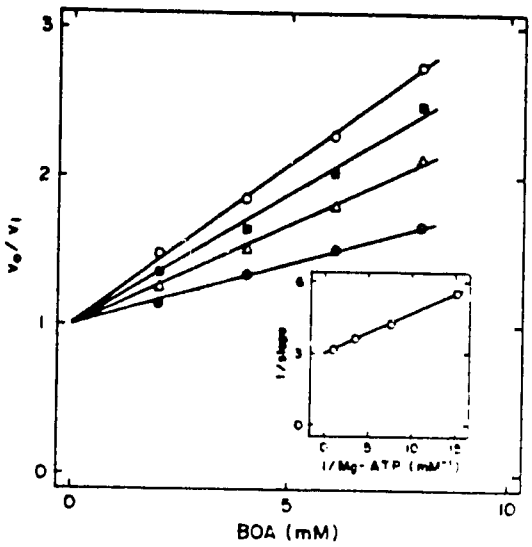

Fig. 3. Uncompetitive inhibition by BOA of ATPase activity of soluble mitochondrial ATPase. Experimental conditions were as described under Materials and Methods. Mg-ATP concentrations were 0.065 (●), 0.130 (△), 0.260 (■) and 1 (○) mM. In the figure, v_0/v_i values are plotted against BOA concentrations, with v_0 equal to the velocity in the absence of inhibitor and v_i equal to the velocity in the presence of the corresponding concentration of inhibitor. In the inset, the reciprocal of the slopes of the v_0/v_i vs (BOA) lines are plotted against the reciprocal of Mg-ATP concentration, according to the procedure described by Roveri (1985) for linear uncompetitive inhibitors. The value for K was determined from the intercept on the y-axis as 3 mM.

and related substances (Vallejos and Stoppani, 1967; Boveris and Stoppani, 1970). However, a difference must be pointed out. BOA inhibited ATP synthesis

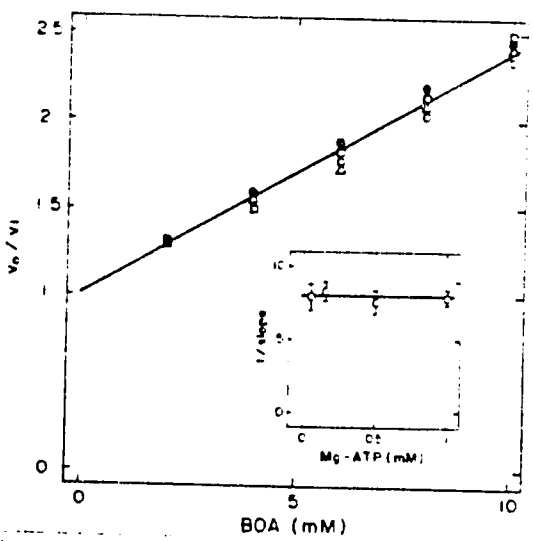

Fig. 4. Non-competitive inhibition of ATPase activity in SMP. ATPase activity was measured as described under Materials and Methods at 0.05 (□), 0.150 (●), 0.50 (○) and 1 (△) mM MgATP. The figure shows the line correlating all the points simultaneously since the slopes of lines corresponding to different substrate concentrations did not differ significantly (see inset). The plot shown in the inset identifies a non-competitive inhibitor, as described by Roveri (1985). The points correspond to the slopes and the bars to the standard deviation of the slopes, obtained by linear regression analysis of v_0/v_i values as a function of (BOA) at the different particular Mg-ATP concentrations.

by acting directly on the ATPase complex and not in the energy coupling.

Finally, the effects of BOA on mitochondrial electron transport from FMN to ubiquinone and on ATPase complex would result in an impairment of ATP synthesis in organisms largely dependent on oxidative phosphorylation for their energy requirements, thus providing a possible explanation for reported deletereous effects caused by BOA.

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GLYCINE-BETAINE ACCUMULATION IN WATER STRESSED BARLEY REDUCED THE EFFECTS OF GRAMINE ON THE APHIDS *SCHIZAPHIS GRAMINUM* AND *RHOPALOSIPHUM PADI*

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Key Word Index -Water stress, aphids, betaine, proline, barley, gramine, *Hordeum*, *Schizaphis graminum*, greenbug, *Rhopalosiphum padi*.

Abstract- Gramine content in different barley cultivars was not changed under water stress. Proline and glycine-betaine increased as plant water potential decreased. The population growth rate of the aphids *Schizaphis graminum* and *Rhopalosiphum padi* were increased in plants with higher glycine-betaine/gramine ratio. When the aphids were fed with artificial diets with different ratios of glycine-betaine/gramine, the survival of aphids increased in proportion to the glycine-betaine content in the diets. Proline had not effect on the survival of aphids. The LD_{50} of gramine was 0.8 and 4.1 mM when 0 and 12 mM glycine-betaine were added to the diets. These results suggest that glycine-betaine accumulation due to water stress may reduce the deletereous effects of gramine on the aphids *S.graminum* and *R.padi*.

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INTRODUCTION

The identification of factors that may regulate plant-insect interactions may be important for breeding pest-resistant cultivars. For example, gramine and related indole alkaloids are responsible for toxicity of *Phalaris sp* to ruminants (1,2). In addition it has been reported the role of gramine as a factor in the resistance of several barley cultivars to the aphids *Schizaphis graminum* and *Rhopalosiphum padi* (3,4). Gramine concentration in barley shoots declines with age in well-watered plants grown at moderates temperatures (5). On the other hand, gramine content in barley plants under water stress does not change (6). However , barley leaves may accumulate proline and glycine-betaine in response to water stress (7). We have previously reported that accumulation of glycine-betaine in water stressed barley may increased its susceptibility to the aphid *S.graminum*,(8). In this work we report that in barley cultivars that contain gramine, the accumulation of glycine-betaine in response to water stress may reduce the deleterious effects of gramine, increasing the aphid population on the plants.

RESULTS

Proline, glycine-betaine and gramine in plants under water stress.

Measurements of leaf water potentials showed that gramine content remained nearly unchanged in water stressed and unstressed leaves(Table I). However, proline and glycine-betaine content were higher in stressed leaves of barley. Accumulation of proline, glycine-betaine and gramine in leaves depended also on the cultivars used.

Effects of water stress on barley susceptibility

We have previously shown correlations among natural accumulation of glycine-betaine and plant susceptibility to *S. graminum* in a gramine free barley cultivar(8). To determine the effects on aphids produced by gramine and glycine-betaine in stressed plants, seedlings of different barley cultivars were infested with adult apterous nymphs of *S. graminum* and *R. padi*. The population growth rate of aphids on the more stressed plants was lower in those cultivars with higher gramine content (Fig 1). However, in stressed barley seedlings, it was observed that the population growth rate of both species of aphids increased with respect to the normal seedlings. Since proline did not show substancial effects on *S. graminum* (8) it is likely that the observed effects are due to glycine-betaine accumulation. Soluble sugars in both treatments did not change significantly (not shown).

Effects of gramine and glycine-betaine on aphids.

Gramine decreased survival of *S. graminum* and *R. padi* with a similar LD_{50} (0.8 mM)(3,4). When the aphids were fed in diets with a one gramine concentration and different glycine-betaine

concentrations, the survival of both species of aphids increased (Fig 2). These results suggested that glycine-betaine reduced the deletereous effects of gramine on the insects. Proline was not effective in reducing gramine effects on aphids (not shown). The LD_{50} of gramine for S.graminum fed with artificial diets increased in proportion to the glycine-betaine content in the diets(Fig 3). This effect was observed on aphids with gramine and glycine-betaine at concentrations normally found in plants.

DISCUSSION

The results shown here and the ones previously reported (8), suggested that glycine-betaine accumulation may increase the susceptibility of barley seedlings to aphids. In general, it has been assumed that glycine-betaine is a solute involved in osmotic adjustements (9). This compound has been shown to be a major osmoticum in halophilic organisms (10). Moreover, glycine-betaine prevents the inhibition of several enzyme activities by high concentration of salts (11).

Barley may be an important crop in some dry areas (12). In periods of water deficit barley accumulates proline and glycine-betaine (7). It has been proposed that stress-induced glycine-betaine accumulation is an adaptative metabolic response (13,14), and that its accumulation could be a valuable metabolic criterion in breeding for drought resistance (15). However, in spite of the possible beneficial effects of glycine-betaine on plant metabolism under water stress, the results shown here indicated that a higher accumulation of this compound may also

increase the susceptibility of barley to aphids in cultivars with or without gramine. Proline, another metabolite involved in responses of plants to stress, did not change the effects of gramine. Since gramine content does not change with water stress and high glycine-betaine content increases the susceptibility of plants to aphids, it could be possible that plants that accumulate none or small amounts of glycine-betaine are more resistant to aphids that those plants without or reduced concentration of gramine and high glycine-betaine content.

The mechanism involved in insect protection against gramine by glycine betaine is unknown. However, gramine has inhibitory effects on mammalian mitochondria and submitochondrial particles (16). Respiration of the bacterium *Pseudomonas syringae* was also inhibited by gramine. Glycine-betaine totally released this inhibition (17). Thus, it could be possible that glycine-betaine may somehow affect cellular respiration of aphids.

Experimental

Plant material and infestation with aphids. Three barley cultivars were analized (F.Union, Cruzat and MCU-34). Seeds were sown in pots filled with soil. Plants were grown under continuous light at 25° in a growth chamber. Six day-old plants of each cultivar were watered daily with different amount of water for six days. Plant water potentials were measured at this stage of development using the methodology described (17). Each group of plants was infested with adult apterous aphids of *S. graminum* and *R. padi*. The increase in the insect population was determined after six days and population growth rate was

calculated (Population growth rate= $\ln(N_f/N_i)/\Delta t$).

Analyses of compounds. For proline analyses, leaf samples were homogenized in 10 ml of 3 % aqueous sulfosalicylic acid and quantified by the method described by Bates et al (19). Glycine-betaine was quantified by a method described by Grieve et al (20). Dried plant material (0.5 g). was shaken with 20 ml of deionized water for 24 hr at 25° and then filtered. The extract was diluted 1:1 with 2 N H_2SO_4. Aliquots of 0.5 ml were cooled in ice water for 1 hr and then 0.2 ml of cold $KI-I_2$ was added. The tubes were stored at 4° over night and then centrifuged at 10000 g for 15 min. at 0°. The supernant was discarted and the precipitate was disolved in 5 ml 1.2 dichloroethane. After 2.5 hr the absorbance was measured at 365 nm.

Gramine measurements were perfomed as described previously (4). Sugars were extracted in 10 ml of 80 % (v/v) ethanol at 25° with constant shaking. Total soluble sugars were quantified as described by Riazi et al (21). 0.1 ml of alcoholic extract were reacted with 3 ml of freshly prepared anthrone in a boiling water bath for 10 min. The reaction was stopped in ice and the absorbance of the samples was measured at 625 nm

Feeding assays. Assays were perfomed with diets placed between two layers of Parafilm M (22). The diet was a solution containing 35 % sucrose,aminoacids and mineral salts adjusted to pH 6.0. Three samples of ten aphids were used for each treatment.

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TABLE I. Effects of water stress on gramine, proline and glycine-betaine content in barley seedlings.

CULTIVAR	Water potential (-bars)	COMPOUND (mmoles /kg dry wt)		
		gramine	proline	glycine-betaine
F.Union	5.3	N.D.	8 ± 1	18 ± 2
	12.0	N.D.	30 ± 3	90 ± 8
Cruzat	5.6	20 ± 2	20 ± 2	54 ± 4
	12.0	19 ± 2	31 ± 3	94 ± 8
MCU-34	5.6	37 ± 3	7 ± 1	31 ± 2
	12.0	30 ± 3	37 ± 3	50 ± 4

Barley seedlings, were grown in soil under continuous light in a growth chamber at 25± 2°. Two groups of six day- old seedlings were irrigated daily with different amounts of water for six days. Leaf water potentials were measured in twelve day-old plants using a pressure chamber. Each value is the mean of two samples + 1 S.E. The compounds were analyzed as described in

FIGURE LEYENDS

Fig. 1 Water stress and susceptibility of barley to aphids

Barley seed cv F.Union,Cruzat and MCU-34 were grown in soil under continuous light in a growth chamber at 25 +2°. Six day-old seedlings were subjected to water stress as described in Experimental.Each of these plants were infested with 2 nonalate adults.Aphids were counted after 6 days. Each value is the mean of three samples of ten plants each. Vertical bars are 1 s.e. Population growth rate = ln (Nf/Ni)/Δt. (day^{-1})

Fig. 2 Effects of glycine-betaine of survival of aphids fed in diets with gramine

Aphids were fed with artificial diets with 3 mM gramine and glycine-betaine at various concentration. Survival was measured after 48 hr. Each value is the mean of three samples of ten aphids . Vertical bars are 1 s.e.

Fig. 3 Survival of S.graminum in diets with gramine and glycine-betaine

Adult aphids were fed with artificial diets containing various amount of gramine and glycine-betaine. Survival was measured after 48 hr (3-A). The numbers over the curves indicate glycine-betaine concentration (mM). The lethal dose of gramine as affected by glycine-betaine was then calculated (3-B).

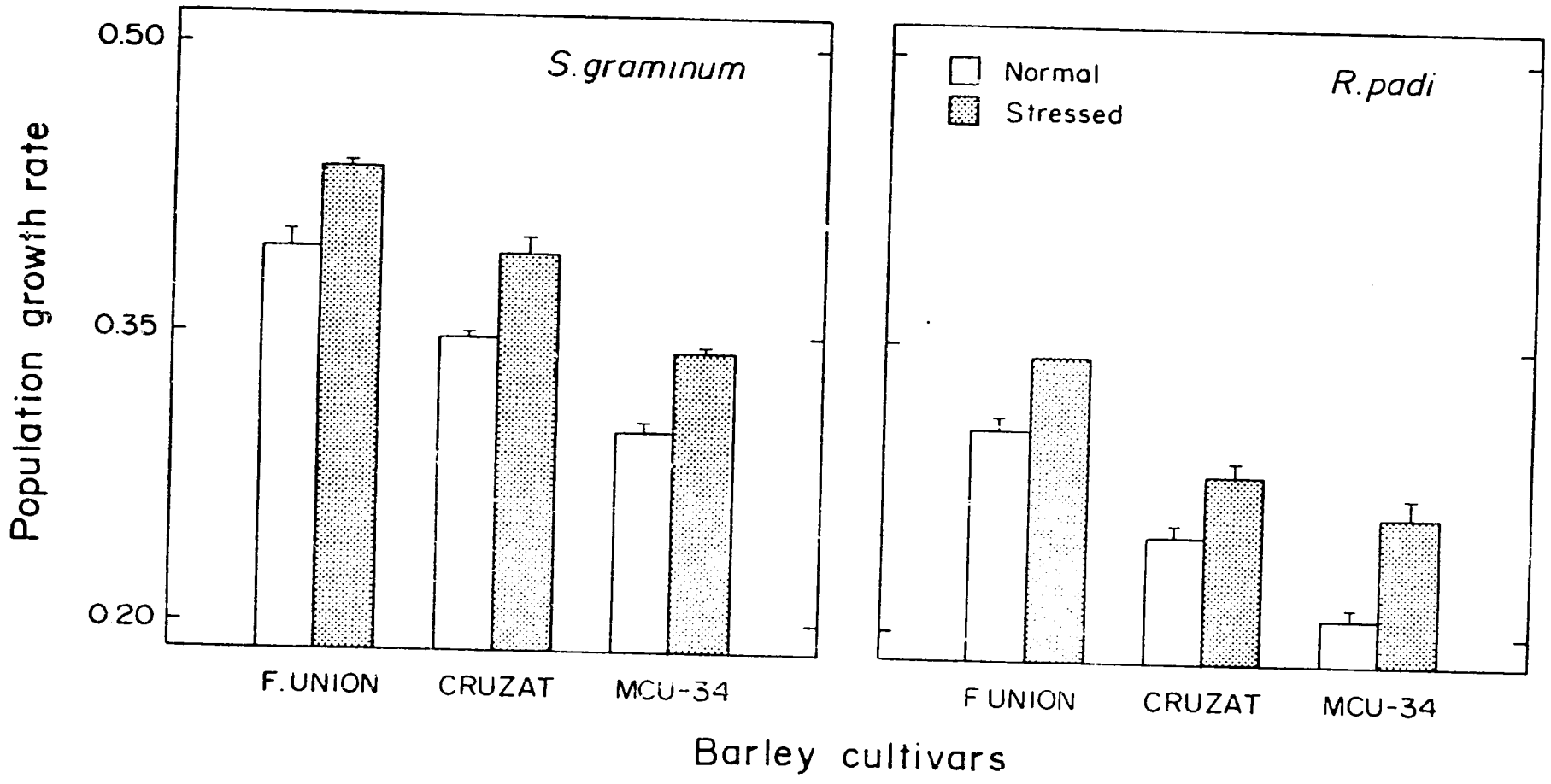

Fig 1
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Fig 2
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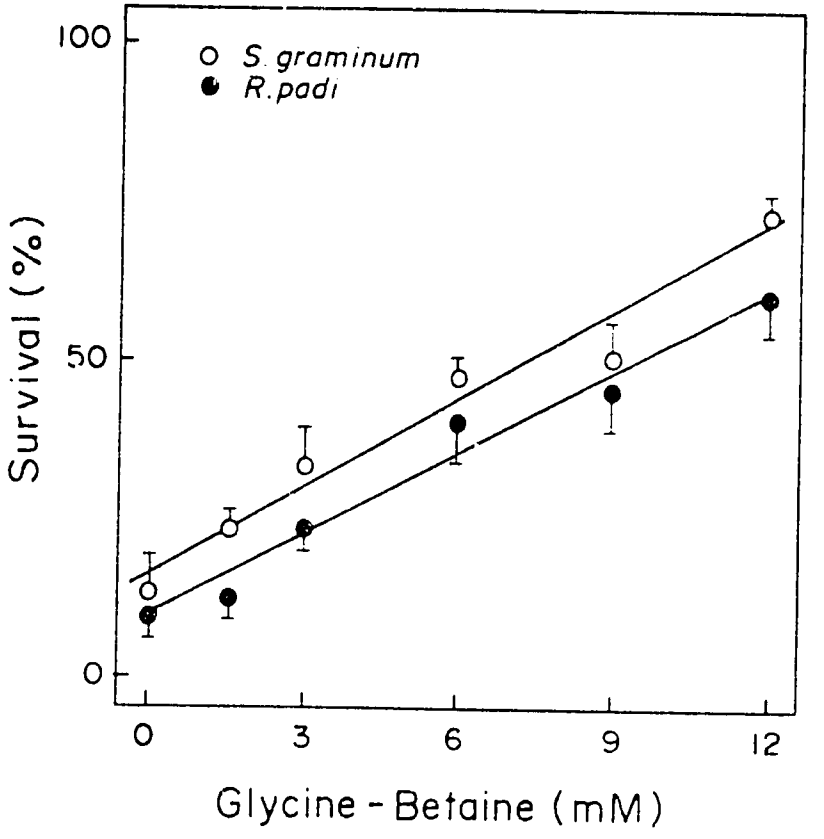

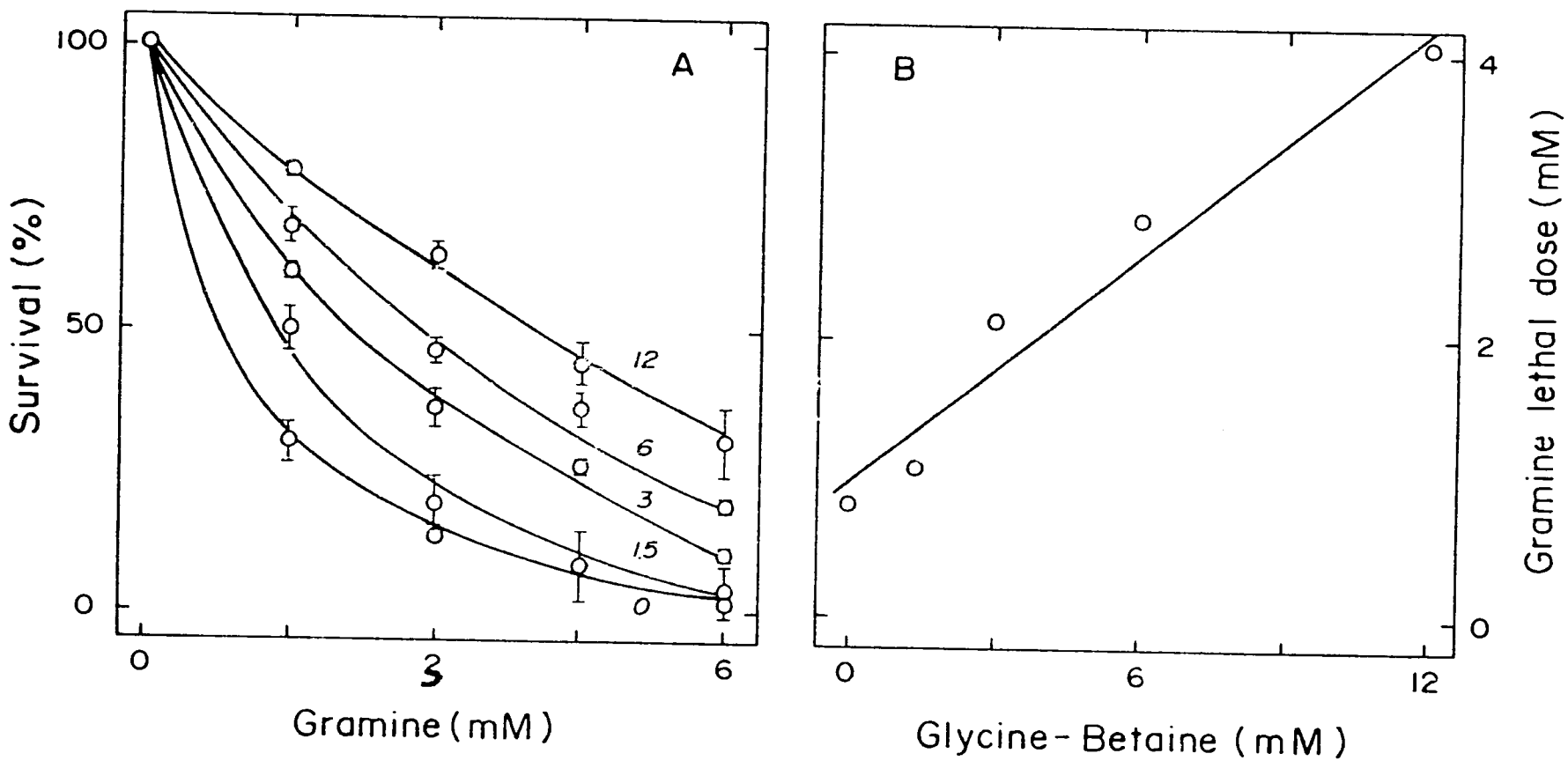

Fig 3
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DISTRIBUTION OF GRAMINE AND HYDROXAMIC ACIDS IN BARLEY AND WHEAT LEAVES

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(*Revised received* 25 *November* 1986)

Key Word Index—*Hordeum, Triticum, Schizaphis graminum*, greenbug, aphids, gramine, DIMBOA

Abstract—The first leaf of 12-day-old barley seedlings, contained 1.3 μmol/g fr. wt gramine while the concentrations were 2.2, 2.0 and 3.1 μmol/g fr. wt in lower and upper epidermis and mesophyll parenchyma, respectively. Gramine was not detected in the vascular bundles, nor in xylem exudates or guttation drops. Thus, about 70% of total gramine in the leaf was found in the mesophyll parenchyma and 30% in epidermal tissue. The content of hydroxamic acids was 3.0 μmol/g fr. wt in the first leaf of 12-day-old wheat plants, while the concentrations were 6.8 and 4.1 μmol/g fresh wt in the vascular bundle and mesophyll parenchyma, respectively. These compounds were not detected in epidermis, xylem exudates and guttation drops. About 50% of total hydroxamic acids were found in the vascular bundles. The significance of these results in plant protection against aphids is discussed.

INTRODUCTION

Secondary metabolites may protect plants against insects and pathogens [1]. Quinolizidine alkaloids from the Fabaceae may be involved in plant resistance to the broom aphid *Aphis cytosorum* [2] and in allelopathy [3]. The concentration of these compounds varies among tissues of the plant with marked diurnal fluctuations [4, 5]. Dhurrin is located in the epidermal layers of the leaf blade of sorghum [6]. Thus, to assign a defensive role to a compound, the location and concentration of the compound in the plant should be considered, in addition to the toxic and feeding deterrent properties of that compound. Gramine, a simple indole alkaloid, is a constituent of barley leaves [7]. The major secondary compounds in wheat are hydroxamic acids [8]. It has been suggested that gramine and hydroxamic acids are involved respectively in resistance of barley and wheat to aphids, [9–12]. In this paper we study the distribution of these compounds in tissues of barley and wheat leaves.

RESULTS AND DISCUSSION

Barley leaves were analysed at various ages for gramine content. Younger leaves always had a higher concentration of this compound (Table 1). Gramine in barley and hydroxamic acids in wheat were heterogeneously distributed among leaf tissues (Table 2). Since only 33% of total gramine present in a barley leaf was in the epidermal tissues and it was not detected in the vascular bundles, the rest should be found in the parenchyma cells of the mesophyll. For hydroxamic acids about 50% were present in the vascular bundles and were not detected in the epidermis. Thus, the other 50% should be found in the parenchyma cells of the mesophyll. To test this hypothesis, mesophyll protoplasts were obtained from wheat and barley. These protoplasts were then analysed for gramine and hydroxamic acids. As expected, both gramine and hydroxamic acids were found in high concentrations in mesophyll protoplasts of barley and wheat, respectively (Table 3).

Gramine has been reported to have toxic and feeding deterrent properties against aphids [12]. Since gramine is not present in the vascular bundles, a preferred feeding site of *Schizaphis graminum*, its effects in protecting barley plants against this aphid may be a consequence of the deterrent properties of the compound during penetration of the leaf by the stylet and subsequent probing before reaching phloem. On the other hand, hydroxamic acids are present in the parenchyma cells of the mesophyll and in the vascular bundles, and it is more likely that the aphids would ingest these compounds. The presence of gramine in epidermal tissues may be relevant to protection of barley against other insects and plant pathogens.

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EXPERIMENTAL

Separation of tissues. Plants grown at 25° with a 14 hr photoperiod were used to isolate leaf tissues. Vascular bundles were separated by using carborundum as described in ref. [13] and then by mechanical separation with a dissection needle under a microscope. The lower epidermis was obtained by storing the leaves at −15° for 5 min, two times with an interval of 3 min. Then, the leaves were put on a cold glass with the upper epidermis on the glass surface. The lower epidermis was moistened with H_2O using a soft brush and mechanically separated. Upper epidermis was obtained from the same leaf after removing lower epidermis, veins and parenchyma cells with a dissection needle and a fine brush. The upper epidermis remained on the glass.

Protoplast isolation. Protoplasts were prepared from the mesophyll of primary leaves of barley seedlings (*Hordeum vulgare* var. × 81-T-1027) and wheat seedlings (*Triticum aestivum* L. var. SNA-3) grown for 12 days in soil. The excised surface-sterilized leaves (4 g) were cut in pieces (about 1 mm) and then incubated in 40 ml of medium containing 1.5% cellulase (Calbiochem), 0.55 M mannitol (pH 5.6) at 28° and gently shaken for 3 hr. Leaf residues were removed through an 80 μm nylon net and protoplasts were purified as described in ref. [14]. These protoplasts were used for quantification of gramine and hydroxamic acid in barley and wheat, respectively.

Quantitation of hydroxamic acids. Tissues were ground in water using a mortar and pestle, filtered through cheese cloth and left for 15 min at room temp. The extract was adjusted to pH 3 with M HCl and centrifuged at 5500 *g* for 15 min. The supernatant was extracted into Et_2O (2:1) and the organic phase was evaporated to dryness. Hydroxamic acids form a blue complex upon addition of $FeCl_3$ reagent (50 g $FeCl_3$ $6H_2O$, 500 ml of 95% EtOH and 5 ml of 11 M HCl). The concentration of hydroxamic acids was determined by comparing the *A* of the extracts with a standard curve made with DIMBOA, the main hydroxamic acid from wheat (ε_{590} = 1315). Thus, the values represent DIMBOA equivalents [8, 15].

Quantitation of gramine. Plant tissues were frozen, homogenized in a mixture of MeOH–NH_4OH (100:1) filtered and evaporated to dryness. This extract was solubilized with 0.1 M HCl. After adjusting to pH 9, the alkaloids were extracted with $CHCl_3$ (1:2). This extract was used for quantitation of gramine with Ehmann's reagent for indoles, as described in ref. [11, 16]. Gramine was the only compound detected in the extract with Ehmann's reagent.

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Table 1. Gramine concentration in barley leaves at various plant ages

Leaf	Gramine (mmol/kg fr. wt) 9-day-old	15-day-old	30-day-old
First	1.10 ± 0.05	0.90 ± 0.08	0.20 ± 0.05
Second	—	1.60 ± 0.06	0.30 ± 0.04
Third	—	—	0.30 ± 0.04

Barley var. × 81-T-1030 was grown at 25° with a 12 hr photoperiod. Leaves are ordered from the oldest (first to appear) to the youngest. Values represent the mean ± s.e. of 3 samples.

Table 2. Tissular distribution of gramine in barley and hydroxamic acids in wheat

Plant (compound)	Compounds: mmol/kg fr. wt	mmol/kg dry wt	amount per leaf (mmol)
Barley (gramine)			
Complete leaf	1.3 ± 0.1	14.1 ± 0.1	1.5×10^{-4}
Lower epidermis	2.2 ± 0.1	44.6 ± 0.8	2.1×10^{-5}
Upper epidermis	2.0 ± 0.1	39.2 ± 0.8	2.3×10^{-5}
Vascular bundles	N.D.	N.D.	
Wheat (hydroxamic acids)			
Complete leaf	3.0 ± 0.5	37.6 ± 0.1	3.4×10^{-4}
Lower epidermis	N.D.	N.D.	—
Vascular bundles	6.8 ± 0.4	40.7 ± 5.0	1.7×10^{-4}
Guttation drops	N.D.	N.D.	—
Xylem exudates	N.D.	N.D.	—

Barley (var. × 81-T-1027) and wheat (var. SNA-3) leaves of 12-day-old plant grown at 25° were used for the analyses as described in the methods section. Data are the mean of four replicates ± s.e. The total amount of compound present in tissues of one leaf was obtained from the concentration of the compound based on fr. wt and the total wt of the tissue in one leaf. N.D., not detected.

Table 3. Gramine and hydroxamic acids concentration in mesophyll protoplasts of barley and wheat

	Gramine (mmol/kg fr. wt)	Hydroxamic acids (mmol/kg fr. wt)
Complete leaf	1.3 ± 0.1	3.7 ± 0.3
Mesophyll protoplasts	3.1 ± 0.2	4.1 ± 0.5

Leaves of 12-day-old barley (var. × 81-T-1027) and wheat (var. SNA-3) were used to isolate protoplasts as described in methods. About 10^6 protoplasts were used to analyse for gramine in barley or hydroxamic acids in wheat. Values are the mean of four replicates ± s.e.

HYDROXAMIC ACID CONTENT OF *TRITICUM* SPECIES

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INDEX WORDS

Triticum, hydroxamic acids, 1,4-benzoxazin-3-ones, plant resistance, wheat breeding

SUMMARY

Fifty-five accessions of *Triticum* species were analyzed for content of hydroxamic acids (Hx), a natural resistance factor against various organisms. Hx were found in all accessions analyzed. Extreme values were found in wild diploid species: highest in *T. speltoides* (16.0 mmol/kg fr. wt) and lowest in *T. tauschii* (0.21). Modern polyploid wheats sharing the same genome did not show substantial variations in Hx levels. The data suggest possible sources of high Hx levels for wheat breeding programs.

INTRODUCTION

Host plant resistance plays a key role in the control of pest populations in crops. Extracts of cereals such as wheat, maize and rye contain hydroxamic acids (Hx, 1)

which are claimed to be involved in resistance of wheat to stem rust, Puccinia graminis (ELNAGHY & LINKO, 1962), and to the aphids Metopolophium dirhodum (ARGANDOÑA et al., 1980, 1981), Schizaphis graminum (ARGANDOÑA et al., 1983) and Sitobion avenae (BOHIDAR et al., 1986) and to contribute to resistance of maize to fungal disease (BEMILLER & PAPPELIS, 1965; LONG et al., 1975; TOTH TOLDI, 1984), and to insect attack (KLUN et al., 1967; LONG et al., 1977; BECK et al., 1983).

In maize, breeding for high Hx levels has been suggested (KLUN et al., 1970; CABULEA et al., 1977; GAHUKAR, 1979; KOSTANDI et al., 1981), and the inheritance of Hx and sources of suitable parental material have been studied (DUNN et al., 1981; SIMCOX & WEBER, 1985).

We herein report Hx levels of several Triticum species and suggest sources of high Hx levels for wheat breeding programs.

MATERIALS AND METHODS

Plant material. Seed samples were obtained from USDA--Beltsville and from INIA-Chile. Plants were grown in a greenhouse under permanent light at ca. 26° with an 8° range, and harvested at different times according to the experiment.

Quantitation of hydroxamic acids. The method employed was essentially as described (BOHIDAR et al., 1986). Plant

tissue (0.2 to 1.5 g) was macerated with mortar and pestle in water (3 x 2 ml), filtered through cheesecloth and left 15 min at room temperature. The extract was adjusted to pH 3 with 1 M HCl and centrifuged at 10,000 g for 10 min. The supernatant was extracted with Et_2O (3 x 6 ml) and the organic phases evaporated to dryness. Ferric chloride reagent was added (3 ml) and absorbance was measured at 590 nm. Hx concentration was obtained by comparison with a standard curve made with DIMBOA. Values reported are averages of at least 3 replicate determinations which agreed within 10 per cent.

Hx levels determined by this method have been shown to closely correspond to the sum of individual hydroxamic acids determined by specific methods (WOODWARD *et al.*, ZUÑIGA *et al.*, 1983).

RESULTS

Table 1 shows Hx content data for the accessions analyzed. Typical determinations of Hx levels are shown in Table 2 for *T. compactum*.

Hx levels in wheat vary with the developmental stage of the plant (ARGANDOÑA *et al.*, 1981). The variation of Hx levels at 2 plant ages was determined for a few of the accessions studied (Table 3). The results show that decrease of Hx levels were comparable in all the accessions studied, with the exception of *T. monococcum*.

For the comparisons in Tables 1 and 2, ten-day-old seedlings were analyzed, since at that age variation of Hx with age is smaller than in younger seedlings, but still reasonable amounts of tissue for analysis may be readily obtained.

DISCUSSION

Development of maize varieties with increased host plant resistance based on breeding for higher Hx levels has been suggested (KLUN *et al.*, 1970; CABULEA *et al.*, 1977; GAHUKAR, 1979; KOSTANDI *et al.*, 1981). Similar suggestions may be put forward for wheat resistance. In this case, availability of suitable germplasm must first be evaluated.

T. speltoides showed the highest Hx level among the accessions analyzed (Table 1). Interestingly, resistance towards biotype E greenbug (*S. graminum*) in wheat streak mosaic virus-resistant wheat lines was claimed to be derived from *T. speltoides* (TYLER *et al.*, 1985). Furthermore, persistance of Hx in older plants of *T. monococcum* (Table 1) may be related to the higher resistance to the aphids *M. dirhodum* and *S. avenae* reported, as compared with accessions of *T. dicoccum*, *T. spelta* and *T. aestivum* (SOTHERTON & VAN EMDEN, 1982).

Modern wheats are amphipolyploids containing different combinations of genomes G, A, B, and D, as

shown in Fig. 1 (SCHMIDT, 1974; BRIGGLE, 1980). It may be suggested from Fig. 1 that the unknown wild diploid species contributing genome B may contain high Hx levels. It would be desirable to test this prediction on *T. searsii*, recently claimed as the B genome donor (THOMPSON & NATH, 1986). Additionally, the present results indicate that substantial increases in Hx levels are unlikely to be attained by hybridization within a set of wheats sharing the same genomes. Furthermore, they show that largest differences in Hx levels occur upon changes of ploidy. It would seem desirable to produce amphipolyploidy utilizing the wild species possessing the G and B genomes (Fig. 1).

Wild relatives of wheat have been extensively used as genetic sources for resistance towards various organisms through wide hybridization (SHARMA & GILL, 1983). Hence, it would be desirable to search for sources of high Hx levels in species of the subtribe Triticinae, in which intergeneric hybridization with wheat is readily achieved (DEWEY, 1984). This approach has already shown promise: a screening of several wild Gramineae showed that *Elymus gayanus* contains relatively high Hx levels (ZUÑIGA *et al.*, 1983).

Hydroxamic acids are involved in the detoxification of atrazine-derived herbicides (HAMILTON, 1964). Hence, in addition to increasing host plant resistance to fungal

and insect attack, an increase in Hx levels could bring increased differential tolerance to these herbicides.

Finally, it should be pointed out that an increase in the level of toxic hydroxamic acids in wheat pose no threat to human consumption, since Hx are not present in the grain (ARGANDOÑA *et al.*, 1981).

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Table 1. Hydroxamic acid content of *Triticum* species[a]

Species	n[b]	Range of Hx (mmol/kg fr. wt)	Mean Hx (mmol/kg fr. wt)
T. aestivum	4	0.69 - 2.0	1.4
T. araraticum	2	2.1 - 3.5	2.8
T. boeoticum	1	0.52	0.52
T. carthlicum	3	3.5 - 4.5	3.8
T. compactum	5	1.2 - 2.8	1.9
T. dicoccum	4	4.1 - 6.1	5.1
T. durum	2	3.6 - 4.9	4.2
T. macha	2	1.6 - 2.5	2.0
T. monococcum	4	0.26 - 1.7	0.62
T. polonicum	3	3.8 - 6.2	5.3
T. spelta	5	1.6 - 3.4	2.5
T. speltoides	5	12.4 - 16.0	14.3
T. sphaerococcum	4	2.6 - 3.4	2.9
T. tauschii	3	0.21 - 0.46	0.37
T. timopheevi	4	1.3 - 2.9	2.5
T. turgidum	4	2.6 - 6.4	4.7
T. zhukovskyi	1	1.8	1.8

[a] Ten-day old seedlings were analyzed.

[b] Number of accessions studied.

Table 2. Hx levels in 10-day-old seedlings of 5 accessions of *T. compactum*

Accession	Origin	n[a]	Hydroxamic acids[b] (mmol/kg fr. wt)
B 237	Turkey	4	1.72 ± 0.13
K192Q x AL	Kenya	4	2.77 ± 0.13
Montsec	Spain	3	1.21 ± 0.12
Compactoide 5	Switzerland	3	2.17 ± 0.11
Vardenin 9	USSR	4	1.44 ± 0.08

[a] Number of replicates.

[b] Mean ± standard deviation.

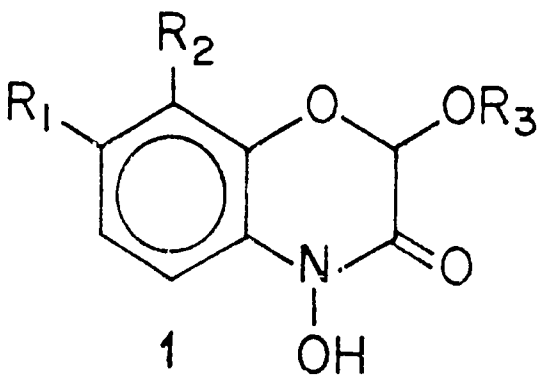
Table 3. Variation of Hx content with plant age in *Triticum* species

Species	PI number	Hydroxamic acids (mmol/kg fr. wt) Plant age 6-day	10-day	Decrease in Hx (%)
T. carthlicum	94754	6.50	3.24	50
T. compactum	352288	5.77	2.23	61
T. macha	428179	5.54	1.95	65
T. monococcum	168804	0.72[a]	0.58[a]	19
T. polonicum	290512	7.87	4.80	39
T. spelta	348474	6.74	3.77	44
T. sphaerococcum	4531-0	4.74	2.26	52
T. turgidum	91673	5.36	2.35	56

[a]Numbers do not differ significantly (P= 0.05).

Legend to Figure

Fig. 1. Mean hydroxamic acid levels along the evolutionary development of wheat. Hexagons= hexaploid wheats; rectangles= tetraploid; truncated circles= diploid..



1

R_1 = H or CH_3O

R_2 = H or CH_3O

R_3 = H or Glucosyl

DIMBOA : $R_1 = CH_3O$, $R_2 = R_3 = H$

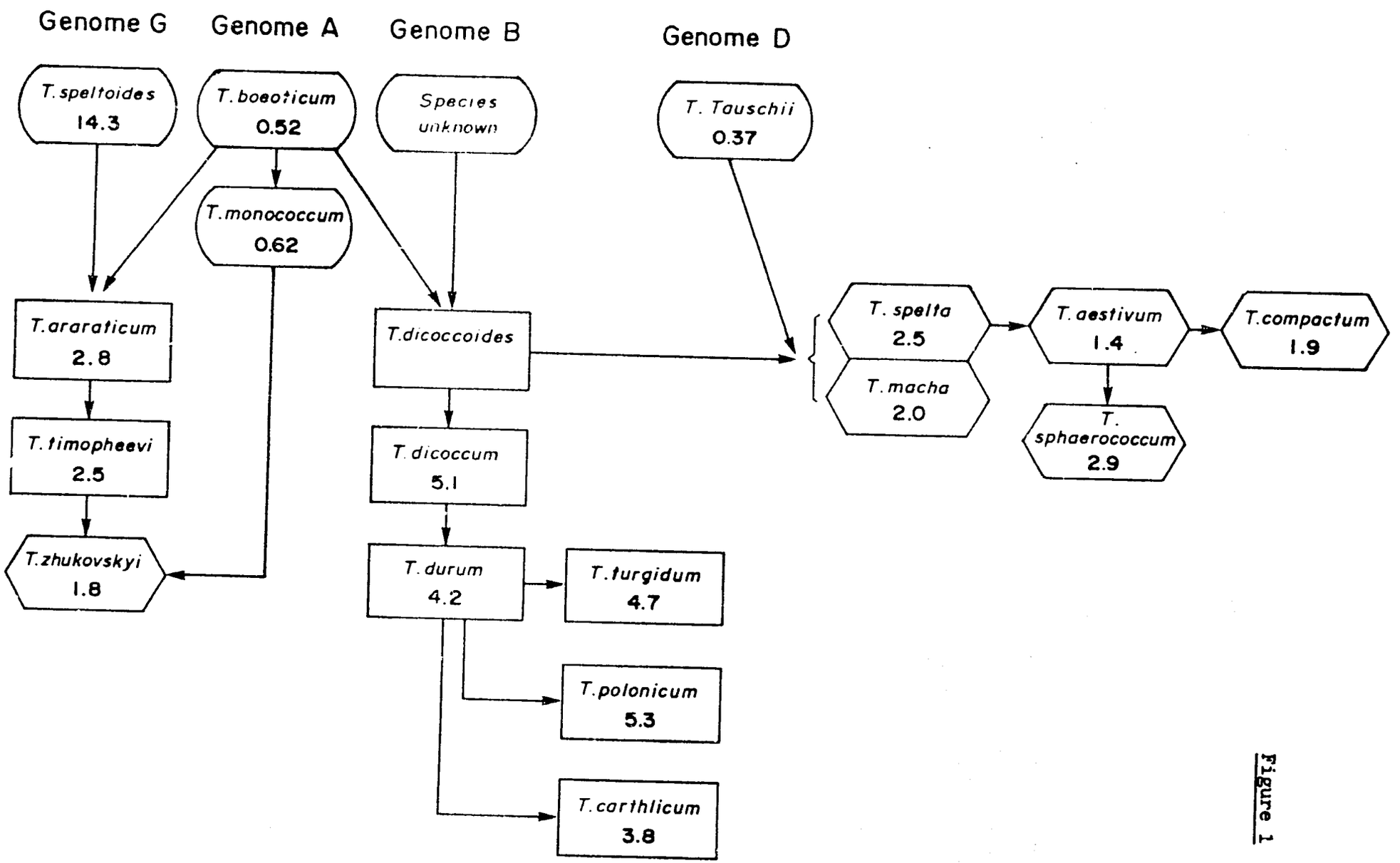

Genome G
Genome A
Genome B
Genome D
T. speltoides
14.3
T. boeoticum
0.52
Species unknown
T. Tauschii
0.37
T. monococcum
0.62
T. araraticum
2.8
T. dicoccoides
T. spelta
2.5
T. aestivum
1.4
T. compactum
1.9
T. macha
2.0
T. sphaerococcum
2.9
T. timopheevi
2.5
T. dicoccum
5.1
T. zhukovskyi
1.8
T. durum
4.2
T. turgidum
4.7
T. polonicum
5.3
T. carthlicum
3.8

Figure 1

INDOLE ALKALOIDS FROM *PHALARIS* AND OTHER GRAMINEAE

Luis J. Corcuera

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I. INTRODUCTION

A variety of compounds that have no recognized role in primary metabolism accumulate in plants(1). These "secondary" compounds are nonetheless, important for the survival of plants since they may be involved in allelopathy, plant-insect interactions, plant toxicity to herbivores and pathogens, and in adaptations to the environment. Alkaloids are one of the most interesting group of compounds due to their use in medicine, allucinogenic properties, and broad biological activities that may confer them important ecological roles. In this article it will be attempted to give an integrated view of the biology and chemistry of the simple indole alkaloids from the Gramineae.

II. CHEMISTRY AND OCCURRENCE IN PLANTS

The simple indole protoalkaloids from *Phalaris spp.* do not contain heterocyclic nitrogen other than in the indole ring. Thus, they can not be considered true alkaloids. Protoalkalois are biological amines formed from similar precursors to the true alkaloids(2). Several of these proto and true alkaloids have been described in Gramineae (Figure 1) and are present in a variety of plant species (Table 1). In addition,

these or other alkylamines derived from tryptophane have been described in other plant families and animals(3,4,5,6,7,8,9).

Gramine [1], the simplest indole protoalkaloid, has raised considerable interest because of its chemical and biological properties. Gramine, a Mannich base has been used extensively in synthesis of indoles and as an alkylating agent(6). Gramine has been chemically synthesized by a Mannich reaction among indole, formaldehyde and diethylamin (10). Radiolabelling of gramine and related alkylamines has been accomplished by chemical synthesis(11)(Figure 2). The importance of gramine and tryptamine derivatives in organic synthesis has been reviewed(6).

III. BIOSYNTHESIS AND DEGRADATION

Gramine and related tryptamine derivatives accumulate mainly in the young leaves. For example, gramine content reaches a maximum during the first two weeks of growth of barley seedlings, decreasing afterwards(12). Feeding studies applying ^{14}C-tryptophane to the leaves indicate that although gramine synthesis takes place mainly at the basal portion in barley leaves, accumulation of this compound is greater at the leaf tip(13).

Tryptophane is the inmediate precursor of tryptamine(13,14,15). A pyridoxal phosphate-dependent tryptophane decarboxylase has been purified from Phalaris tuberosa(14). The activity of the enzyme is inhibited by N,N-dimethyltryptamine, one of the major alkaloids produced by P. tuberosa, suggesting a control mechanism for this biosynthetic route. Then, tryptamine derived alkaloids are synthesized by several N-methyltransferases using S-methyl-adenosyl-methionine as a methyl donor(16,17,18). Several pathways have been proposed for the biosynthesis of the various compounds (Figure 3)(19).

Tryptophane is also a precursor in the biosynthesis of gramine(20,21). The indole ring, the methylene side chain and possibly the amino N are derived from tryptophane without cleavage of the indole-alanine linkage (22,23). The immediate stable intermediates for the biosynthesis of gramine are 3-aminomethylindole (AMI) and 3-methylaminomethylindole (MAMI). These compounds yield gramine upon N-methylation with S-adenosylmethionine in the presence of a crude enzyme extract from barley (Figure 4)(18,24,25). It has been suggested that sequential methylations of AMI could be catalized by two separate enzymes(19). Although the metabolic pathway for the biosynthesis of gramine is firmly established, the mechanism by which tryptophane is converted to AMI remains unclear.

Gramine is rapidly degraded by barley tissues. When

[α-^{14}C]gramine was fed into excised barley shoots, 0.4% of the radioactivity was found in tryptophan, 10% in indole-3-carbinol and 6% into indole-3-carboxylic acid(26,27,28,29). A nearly quantitative degradation of [α-^{14}C]gramine to $^{14}CO_2$ has been found to occur in intact barley plants with no evidence for alternate pathways(30). Since in these experiments gramine is introduced in plant compartments where the compound is not naturally found, it would be of interest to determine whether these degradation products also occur in those tissues where gramine is found in the plant. While gramine is found in epidermal tissues and mesophyll parenquima of barley leaves, it is not found in the vascular bundles(31). Thus, the physiological meanings of these degradations remain unclear.

IV. TOXICITY TO MAMMALS AND PLANTS

Effects on animals. *Phalaris* species are important components of pastures around the world. However, intake of these plants sometimes produces sudden collapse and death, and *Phalaris* staggers, a chronic syndrome in sheep(3,32,33,34). The persistance of neurological signs after removal from the *Phalaris* pastures suggests the presence of degenerative lessions in the central nervous system(34). Gallagher *et al.*(34) have shown that all ages, breeds and sexes of sheep appear to be susceptible, while death of cattle is not common. Peracute poisoning with signs of neurological and cardiac disorder may occur in the same outbreak. Necropsies of sheep suffering this syndrome have shown severe

abdominal visceral congestion, hemorrhages in the epicardium and the heart stopped in systole or diastole(32,34).

It has been proposed that *Phalaris* poisoning syndromes are caused by indole protoalcaloids present in tissues of this plant. Since *in vitro* digestibility is not affected by the alkaloids present in *Phalaris*, poor animal performance with the plant is probably due to factors other than alkaloid interference with rumen microflora(35). It has been suggested that is likely that some pharmacological properties of serotonin may be interfered by indole protoalkaloids because of their structural similarities(32). Gramine, methyltryptamine and 5-methoxydimethyltryptamine caused tachycardia, ventricular extrasystoles, and fibrilation. Parenteral administration of the alkaloids caused profound disturbances of the central nervous system in sheep: hyperexcitability, urination, pupilary dilatation, licking of the lips, head nodding, high rectal temperature, etc. Thus, these compounds appear to be responsible for at least some of the observed symptoms.

Meadow voles (*Microtus pennsylvanicus*) have been used to evaluate forage quality(36,37). Palatability of leaves of tall fescue and reed canarygrass was negatively correlated with the total alkaloid level(38). In short term experiments gramine 5-MeO-N,N-dimethyltryptamine, 5-MeO-tryptamine, N,N-dimethyl-tryptamine and N-methyltryptamine made the diets unpalatable for

meadow voles at concentrations similar to those found in plant tissues(38,39). When the animals were fed 0.25 or 0.5% gramine in the diet for 21 days about one third of the voles died. Those that survived had kidney lesions, glycosuria, higher intakes and lower weight gains than control animals(40). Also, voles fed gramine had increased reticulocyte levels and higher alkaline phosphatase than the control animals. This bioassay system may be useful in judgements as to which chemicals can be tested in the expensive trials with large animals and ultimately included in plant breeding programs(39). Palatabilty to sheep and cattle of cultivars containing various concentrations of alkaloids is consistent with palatability data with the pure compounds fed in diets to voles(38,41). Thus, palatability decreases as alkaloid content increases(42,43). Since the alkaloids cause deleterious effects on various animals, an attempt has been made to reduce the concentration of the compounds in reed canarygrass.It has been found that lamb performance improves when the animals are fed with those cultivars low in alkaloids(44). If the alkaloid concentration is 0.2% of the dry weight or above, the lambs will show reduced weight gains. This study has also shown that a tryptamine-carboline free cultivar does not incite diarrhea, although gramine is still present, being possible that poisoning syndromes may be caused by a variety of compounds. Considering all these deleterious effects on animals, it has been proposed to reduce or eliminate alkaloids in these plants by plant breeding or by cultural practices (19).

Effects on plants. Barley leachates caused inhibition of germination and growth of *Stellaria media*, *Capsella bursa-pastoris* and tobacco and had no significant effect on wheat(45). Gramine, which is present in barley, produced 77% inhibition of growth of *S. media* when added to the nutrient solution at 0.05 mM. However at much lower concentration there was an stimulation of growth. Germination was not affected by 0.05 mM gramine. If these allelopathic properties of barley are relevant under field conditions needs further testing.

Gramine causes phytoxicity on barley itself depending on environmental conditions(19). Growth of barley plantlets was not affected by gramine at 25 C. Nonetheless, when plantlets were kept at higher temperatures growth was decreased significantly by 10 mM gramine provoking chlorosis and necrosis. In contrast, achlorophyllus callus tissue was not affected by endogenous or exogenous gramine. The differential activity of gramine on these tissues could be explained by the effects of gramine on chloroplasts. Gramine inhibited photophoslorilation, Pi-ATP exchange reaction and proton gradient and enhanced electron transport in spinach thylakoids with an I_{50} around 0.2 mM(46). The ATPase activity of soluble coupling factor was not affected. Thus gramine appears to behave as an uncoupler. The apparent behavior of gramine as an uncoupler was also observed in rat and bovine mitochondria, although the effect was much smaller(47). At higher concentrations (I_{50} = 2-6 mM) gramine

inhibited electron transport at the level of complex I of the respiratory chain. Also, an inhibition of coupled succinate oxydation was observed (I_{50} =10 mM). The same effects were observed in the phytopathogenic bacterium *Pseudomonas syringae*(48). The effects of gramine on plant mitochondria remain to be tested.

Gramine may then stimulate biochemical and biological processes at low concentrations and inhibit the same or other processes at higher concentrations. Among these cases are stimulation and inhibition of growth of *Stellaria*, *Pseudomonas syringae*, and *Tetrahymena*(49). It has been proposed that the stimulatory effects of gramine at low concentrations may be due to its similarity with indole-3-acetic acid and tryptophane (45).A more likely explanation is that gramine is catabolized in plants and there appears to be some recycling of the indole nucleus to tryptophan(27,28), but this has not been clearly demonstrated(21). Stimulatory indole compounds could then be formed from tryptophane.

From the previous discussion, it is apparent that presence of gramine in barley and possibly in other plants may cause damage to the plants under certain environmental conditions and also to some animals of economic importance that feed on these plants. If this is the case, selection of low gramine cultivars may be worthwhile. However, gramine and related

protoalkaloids may have ecological importance that should be considered before selecting against these compounds.

V. ROLE IN RESISTANCE TO PATHOGENS AND PESTS

Gramine has a wide variety of biological activities. Among those are toxicity to mammals and plants. In addition, indole protoalkaloids showed toxicity and caused feeding deterrency on the aphids *Schizaphis graminum*, and *Rhopalosiphum maidis* reared on a synthetic diet (50). The LD_{50} of methyl-tryptamine, methoxy-N,N'-dimethyltryptamine, gramine and methoxy-tryptamine on *R. maidis* were 3.8, 3.5, 2.9 and 2.3 mM, respectively. Concentration of gramine in barley ranges from 0-5 mmoles/kg fresh weight (51). Therefore, the effects of gramine on aphids occurr within the concentration range in which gramine is found in plants. When artificial infestation experiments of barley with *Schizaphis graminum* were performed, gramine content of the leaves correlated with resistance to the greenbug *S. graminum*. Similar results were found in infestation experiments performed with barley and *R. padi* (52). Although *S. graminum* and *R. padi* were similarly affected by gramine in artificial diets, cultivars high in gramine were more resistant to *R. padi* than to *S. graminum*. This apparent contradiction could be explained by distribution of gramine in barley leaves and feeding sites of aphids. Gramine is not

homogeneously distributed among tissues in barley leaves(31). About one third of total gramine is found in upper and lower epidermis, two thirds in the mesophyll parenchyma and none in the vascular bundles. Thus, studying the feeding behavior of these aphids in relation to distribution of toxicants in barley leaves would be of importance in assesing the possible role of gramine in resistance of barley to aphids. Other insects on which gramine affects feeding are Scolytus multistriatus(53), Melanoplus bivittanus(54) and Heliothis zea(55). Being gramine located in upper and lower epidermis and parenchyma cells of the mesophyll, could be of importance for protection of the plant against chewing insects and plant pathogens. Nonetheless, this remains to be shown.

VI. CONCLUSIONS AND FUTURE PERSPECTIVES

Indole alkaloids from Gramineae are apparently important in barley, Phalaris and other plants. Concentration of these compounds in plants may depend on cultivar (51,56,57), plant maturity (58,59), cultural practices, and environmental factors (60,61). Since they produce toxic effects in sheep and cattle and perhaps in the plant itself, it may look advisable to select plants low or free from these alkaloids by plant breeding. Undoubtedly this would be benefitial for animal feeding. Nontheless, these alkaloids are also toxic or cause feeding deterrency on a wide variety of organisms that feed or grow on plants. Among these are insects and phytopathogens. Experimental evidence indicates that these compounds may be important in

resistance to these organisms and thus for the survival of the plant. It would be advisable not to implement a selection program to eliminate indole alkaloids from barley and *Phalaris* before alternative resistance mechanisms to pathogens and pests are described and incorporated by plant breeding in the desired cultivars. To do otherwise may produce susceptible cultivars to pathogens and insects with lower biomass or grain yields. Thus, the description of other natural resistance mechanisms to these organisms is eargerly awaited.

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Table 1. Indole protoalkaloids and β-carbolines in several graminaeae

Plant	Compound	References
Arundo donax	Gramine [1]	(3,18,62,63)
	N,N-dimethyl-tryptamine [7]	(3)
	5-Metoxy-N-methyl-tryptamine [8]	(3)
	Bufotenine [9]	(3)
Hordeum vulgare	Gramine [1]	(64)
	3-Aminomethyl-indole [3]	(24)
	3-Methyl-aminomethyl-indole [2]	(24)
Hordeum spontaneum	Gramine [1]	(65)
Hordeum sativum	Gramine [1]	(3)
	N-methyl-gramine [4]	(3)
Phalaris arundinacea	Gramine [1]	(66)
	N-methyl-tryptamine [6]	(66)
	N,N-dimethyl-tryptamine [7]	(66)
	5-methoxy-N-monomethyl-tryptamine [8]	(67)
	5-methoxy-N,N-dimethyl-tryptamine [10]	(66)
	2-methyl-tetrahydro-β-carboline [14]	(68)
	2-methyl-6-methoxy-tetrahydro-β-carboline [15]	(68)
	2,9-dimethyl-6-methoxy-tetrahydro-β-carboline [16]	(68)
Phalaris aquatica (tuberosa)	Gramine [1]	(16)
	N,N-dimethyl-tryptamine [7]	(16,69,70,71)
	5-methoxy-N,N-dimethyl-tryptamine [10]	(16,69,70,71)
	Bufotenine [9]	(70,71)
	5-hydroxy-N-methyl-tryptamine [12]	(70)
	5-hydroxy-tryptamine [13]	(70)
	3-methyl-aminomethyl-indole [2]	(70)
	3-amino-methyl-indole [3]	(70)
	5-methoxy-N-methyl-tryptamine [8]	(70)
	N-methyl-tryptamine [6]	(70)
	5-methoxy-tryptamine [11]	(70)

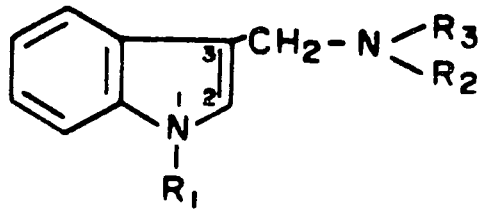
FIGURE LEGENDS

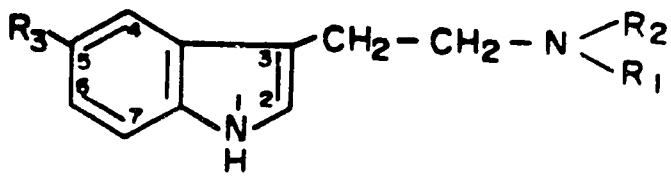
Figure 1. Main indole protoalkaloids and carbolines from gramineae.

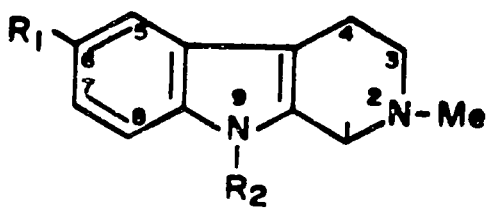
Figure 2. Chemical synthesis of double labelled gramine (11). (*) ^{13}C or ^{14}C; (Δ) ^{13}C or ^{14}C.

Figure 3. Proposed biosynthetic pathway for tryptamine derivatives in *Phalaris tuberosa*. Abbreviations used: TP, tryptophan; T, tryptamine; MMT, N-methyl-tryptamine; DMT, N,N-dimethyltryptamine. Enzymes involved: 1, tryptophan decarboxylase; 2 and 3, N-methyl-transferases.

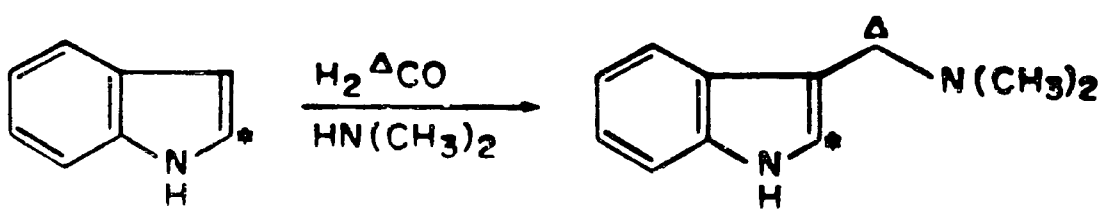
Figure 4. Biosynthesis of gramine in barley leaves.

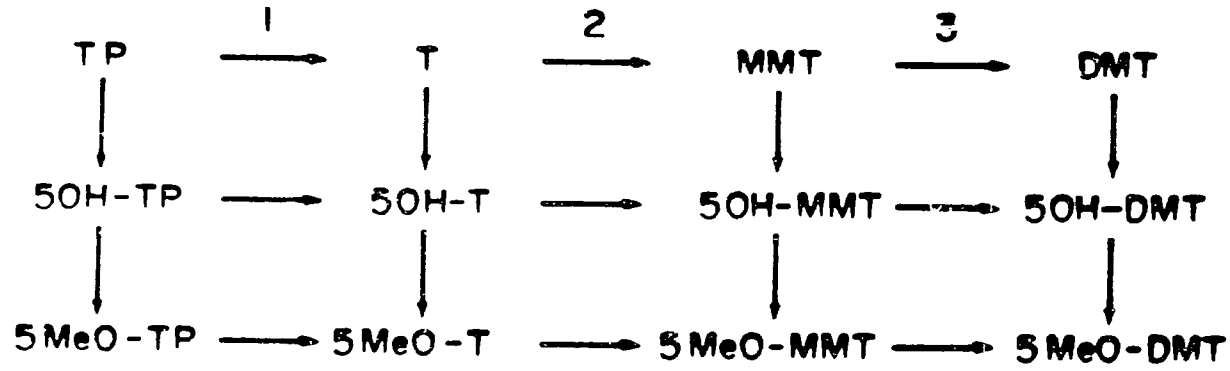






R_1	R_2	R_3	
H	Me	Me	[1]
H	Me	H	[2]
H	H	H	[3]
Me	Me	Me	[4]
H	H	H	[5]
Me	H	H	[6]
Me	Me	H	[7]
Me	H	OMe	[8]
Me	Me	OH	[9]
Me	Me	OMe	[10]
H	H	OMe	[11]
Me	H	OH	[12]
H	H	OH	[13]
H	H	—	[14]
MeO	H	—	[15]
MeO	Me	—	[16]


N
H
H2 ΔCO
HN(CH3)2
Δ
N(CH3)2
N
H


1
2
3
TP
T
MMT
DMT
5OH-TP
5OH-T
5OH-MMT
5OH-DMT
5MeO-TP
5MeO-T
5MeO-MMT
5MeO-DMT

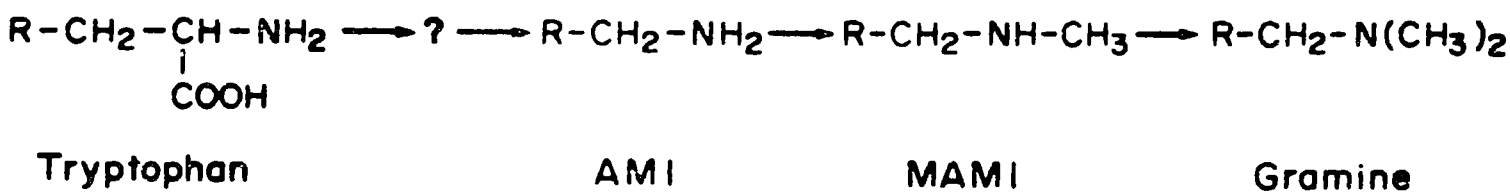

R-CH2-CH-NH2
COOH
?
R-CH2-NH2
R-CH2-NH-CH3
R-CH2-N(CH3)2
Tryptophan
AMI
MAMI
Gramine

Fig. 11