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STUDIES ON LETTUCE SEED GERMINATION—I.

COUMARIN INDUCED DORMANCY

A. M. M. BERRIE, W. PARKER, B. A. KNIGHTS and M. R. HENDRIE

Departments of Botany and Chemistry, University of Glasgow, Glasgow, W.2.

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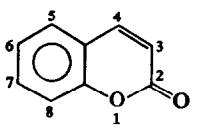
Abstract—A large number of coumarin derivatives and a few isocoumarins were tested with regard to their capacity to induce light sensitive dormancy in seeds of *Lactuca sativa* L. cv. "Grand Rapids". Only those derivatives which are more reduced than coumarin showed this biological activity. Hydroxylation resulted in loss of activity as also happened when coumarin was poly substituted. Mono substitution by methoxyl at positions 4 and 8 did not result in marked reduction of activity. 2-Thiocoumarin was weakly active. The 8-hydroxy-isocoumarin, oosponol had some activity. On the basis of the findings it is proposed that the coumarins and isocoumarins which induce light sensitive dormancy in plants may act as anti-gibberellins. Their action is considered to result from the structural similarity to that moiety of gibberellin containing the lactone bridge. It is supposed that there is competition for the same active site in the plant cell. Competitive inhibition with gibberellic acid is implied from the experimental data in the case of dihydro- and hexahydro-coumarin. With coumarin and 4-methoxycoumarin the data obtained do not allow this interpetation but the inability to demonstrate competitive inhibition may arise because factors like diffusion, bonding and metabolic destruction result in the compounds arriving at the active site in disproportionate amounts. A possible model for the action of gibberellins and anti-gibberellins is proposed.

INTRODUCTION

COUMARIN (I) occurs widely in plants and has been shown to possess biological activity in many different organisms.[1–4] A quite specific type of effect was demonstrated when it was shown that coumarin induced light sensitive dormancy in lettuce seed.[5] Recently ramulosin and patulin have been shown to be capable of inducing this type of dormancy in lettuce.[6,7]



I

There is no knowledge of the mechanism which is brought into play in lettuce seed as a result of treatment by these substances though a suggestion about the role of coumarin is that it uncouples phosphorylation.[8–10] Other effects observed by Mayer and Poljakoff-Mayber are that it interferes with proteinase activity and that courmarin has auxin-like

[1] T. O. SOINE, *J. Pharm. Sci.* **53**, 231 (1964).
[2] A. D. WORSHAM, G. C. KLINGHAM and D. E. MORELAND, *Nature* **195**, 199 (1962).
[3] A. C. LEOPOLD, *Am. J. Botany* **36**, 437.
[4] L. J. AUDUS and J. H. QUASTEL, *Nature* **159**, 320 (1947).
[5] G. E. NUTILE, *Plant. Physiol.* **20**, 433 (1945).
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[7] A. M. M. BERRIE, M. HENDRIE, W. PARKER and B. A. KNIGHTS, *Plant Physiol.* **42**, 889 (1967).
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[9] S. ULITZUR and A. POLJAKOFF-MAYBER, *J. Exptl Botany* **14**, 95 (1962).
[10] A. M. MAYER and A. POLJAKOFF-MAYBER, in *Plant Growth Regulation* (edited by R. M. KLEIN) p. 735, Iowa State University Press. Ames (1961)l.

activity. Coumarin, while not preventing the breakdown of sucrose, did prevent the accumulation of glucose, and also lipase activity was blocked.[11] These reports indicate that coumarin might be operating primarily as an enzyme inhibitor with no marked specificity, though *in vitro* studies to confirm this were without conclusive results.

Blaim examined the effect of coumarin on water uptake by wheat seeds and he found that treated seeds did not take up water to the same extent as untreated seed.[12] The embryo was found to be specifically prevented from imbibing water and it was concluded that the inhibitory effect of coumarin on the germination of wheat was due to this.

It appears that many fundamental processes associated with seed germination might be affected by coumarin and it would be hazardous to make a statement regarding which of these is the first to be affected.

The light sensitivity of naturally dormant lettuce seed or seed in which this phenomenon is induced by exposure to high temperature or coumarin can be broken by gibberellin.[13] Similarly light sensitive dormancy induced by ramulosin can be broken by gibberellin.[6] With patulin there is evidence that gibberellic acid negates its action but there is no statistically demonstrable interaction between them.[7] In terms of physiological effect coumarin and ramulosin can be said to act as anti-gibberellins in the germination of lettuce seed. While the experimental evidence allows this hypothesis, it has not yet been demonstrated that naturally occurring coumarin, or derivatives, have any role to play in the germination of seeds or in the control of organization and development of plants.

RESULTS

Coumarin itself was first tested, and Table 1 shows the response of the particular batch of seed used in the experiments described in this paper. Over fifty coumarin and isocoumarin derivatives have been tested and Tables 2, 3 and 4 present the results of these tests. Coumarin was used as a standard, and the compounds listed are arranged in order of their effectiveness in inducing light sensitivity, and on the degree of substitution on the coumarin nucleus.

Our results on the induction of light sensitivity parallel those obtained by Mayer and Evenari[14] on the effect of coumarins on the germination of wheat and lettuce seeds. From tests on the responses of seed to applications of coumarin and gibberellin we have confirmed that gibberellin is effective in removing the block to germination manifest as light sensitivity but does not overcome the effect which coumarin has on growth of the embryonic axis (see also Mayer[13]).

Activity is retained in the partially reduced dihydro- and hexahydrocoumarins tested, but alkyl or oxygen monosubstituted coumarins either exhibit a much reduced action or have no activity at all.

All di- or polysubstituted coumarins tested were found to be without action in the standard assay, although 3-carbethoxy-4-hydroxycoumarin exhibited the remarkable property of inhibiting root-hair growth, suppressing hypocotyl elongation, and removing the geotropic response of the radicle in seedlings which grow from treated seeds.

Of the mono-unsaturated coumarins assayed, only certain compounds retained activity. Only mono-unsaturated coumarins carrying a nonpolar substituent (e.g. methyl, methoxyl and nitro) retained any degree of action in the standard assay. Herniarin (7-CH_3O) is

[11] A. M. MAYER, Cryptobiotic Stages in *Biological Systems* (1961).
[12] K. BLAIM, *J. Exptl Botany* **11**, 377 (1960).
[13] A. M. MAYER, *Nature* **184**, 826 (1959).
[14] A. M. MAYER and M. EVENARI, *J. Exptl Botany* **3**, 246 (1952).

TABLE 1. A. EFFECT OF COUMARIN ON GERMINATION OF LETTUCE SEED

Condition†	% germination in presence of Coumarin mg/dish			
	0·0	0·01	0·1	1·0
Dark controls	100	54·7	0	0
*Light	100	100	8	0

† Experiment carried out at 20°.
* 4 min W.W.X. fluorescent tube 20 W 3 in. from tube envelope, 4 hr after onset of imbibition.

B. INTERACTION BETWEEN COUMARIN AND GIBBERELLIC ACID ON THE GERMINATION OF LETTUCE SEED

		% germination in presence of G.A. (mg/dish)			
		0	0·01	0·03	0·1
Coumarin mg/dish	0	86	83	83	83
	0·01	66	69	71	71
	0·03	8	24	31	50
	0·1	4	7	1	7

Seed held continuously in darkness at 20° for 48 hr. Replicates, Not significant; Coumarin treatments, $p<0·01$; G.A. treatments, $0·05>p>0·01$; G.A.× Coumarin interaction, Not significant.

TABLE 2. COUMARINS CAPABLE OF INDUCING LIGHT SENSITIVITY IN LETTUCE SEED*

Compound	Activity index†
1. Coumarin	100
2. Reduced coumarins—	
3,4-Dihydrocoumarin (melilotolactone)	100
3,4,5,6,7,8-Hexahydrocoumarin	34·5
3,4,4a,5,6,7-Hexahydrocoumarin	23
3. Monosubstituted coumarins—	
4-Methoxy	100
8-Methoxy	72·8
6-Methyl	58·5
6-Methoxy	52·5
7-Methoxy (herniarin)	29

* χ^2 for dark vs. light in 2 × 2 contingency table > 3·841
† $100-\left(\frac{\text{No. germinated in treated}}{\text{No. germinated in control}}\times 100\right)$

moderately active and it is of interest to note that oxygenation of position 7 is a frequent pattern in coumarins. Compounds containing large (e.g. bromo or benzyl) or polar (e.g. carbomethoxyl, carboxyl and hydroxyl) groups were ineffective, although 3-hydroxycoumarin inhibited germination without rendering the seed light sensitive. In 3-hydroxycoumarin, hydrogen bonding between the lactone carbonyl and the adjacent hydroxyl group will serve

TABLE 3. COUMARINS WITH NO EFFECT ON LETTUCE SEED

Compound	Activity index	Compound	Activity index
1. *Mono-substituted*		2. *Di-substituted* (contd.)	
3-Bromo	5	7-Diethylamino-4-methyl	3
3-Benzyl	8	5,7-Dihydroxy	0
3-Carbomethoxy	0	7-Acetoxy-3-methyl	6
3-Carboxy	1·5	6,7-Dihydroxy (aesculetin)	−3
4-Hydroxy	8	7-Ethoxy-4-methyl	0
7-Hydroxy (umbelliferone)	−2	4-Hydroxy-6-methyl	4
8-Hydroxy	12	7-Hydroxy-6-methoxy (scopoletin)	17·5
3-Methyl	10	7-Hydroxy-4-methyl	3
6-Nitro	5·5	4-Hydroxy-7-methoxy	1
2. *Di-substituted*		4-Methyl-7-acetyl	3·5
3-Acetyl-7-methyl	10	3. *Tri-substituted and miscellaneous*	
3-Amido-7-diethylamino	0	8-Acetyl-7-hydroxy-4-methyl	1
4-Benzyl-7-hydroxy	12·5	8-Benzoyl-7-hydroxy-4-methyl	−2
3-Carbethoxy-4-hydroxy	−1	6-Hydroxy-5,7,8-trimethyl	1
3-Carboxy-6-chloro	−1	6-Hydroxy-glycoside-7-hydroxy	6
3-Carboxy-7-chloro	−1	6,7-Furano-8-methoxy (xanthatoxin)	2·5
3-Carboxy-7-methoxy	−4	7-Acetoxy-3,4-*cyclo*penteno	1
3-Chloro-4-methyl	0	7-Hydroxy-3,4-*cyclo*penteno	1·5
6,8-Dichloro	4		

TABLE 4. THE RESPONSE OF LETTUCE SEED TO ISOCOUMARINS

		Activity index
Ramulosin (V)	Light sensitivity induced	43
Oosponol (VI) $R_1=CO\cdot CH_2OH$, $R_2=H$	Light sensitivity induced	73
Oospoic acid $R_1=COOH$, $R_2=H$	Inactive	1
Oospoglycol $R_1=CHOH\cdot CH_2OH$, $R_2=H$	Inactive	−1
Oospolactone $R_1=CH_3$, $R_2=CH_3$	Inactive	0
4-Methylenecarboxy isocoumarin	Inactive	1·5

to reduce the polarity of the hydroxyl substituent. This substance may also exhibit chelating properties and such properties may account for the observed physiological action.

At high concentrations various disubstituted coumarins will inhibit germination but it is clear that they act differently from such monosubstituted coumarins as are capable of invoking the phytochrome system as a control mechanism in the germination of lettuce seed.

Whilst 2-thiocoumarin is slightly active (activity index at 1 mg/dish = 42·5) no evidence is available to show that 1-thiocoumarin or 1,2-dithiocoumarin possess the specific affects under consideration. Insufficient 1-thiocoumarin was available for a complete assay,

although the results obtained suggest it may have properties similar to those of coumarin, and the dithio compound was unavailable.

In addition to substituting for light in the germination of lettuce seed, gibberellin will reverse the effect of coumarin and also of ramulosin. In Table 5 are shown the results obtained when testing the interaction of gibberellin and some of the more active compounds listed in Table 2 upon lettuce seed. Whilst there is not a striking reversal of the induced dormancy, the results indicate that gibberellin does counter the effect of these compounds on lettuce seed.

TABLE 5. INTERACTION BETWEEN ACTIVE COUMARINS AND GIBBERELLIC ACID

Test subs.	mg/dish	% germination of lettuce in presence of gibberellic acid mg/dish					
		0	0·003	0·01	0·03	0·1	0·3
3,4-Dihydrocoumarin	0·000	90	92	95	95	91	—
	0·003	83	89	93	82	78	—
	0·01	51	69	76	82	86	—
	0·03	12	27	59	41	72	—
	0·1	1	10	15	37	26	—
3,4,5,6,7,8-Hexa hydrocoumarin	0	87	—	88	93	87	87
	0·01	72	—	80	86	79	66
	0·03	47	—	61	65	81	53
	0·1	18	—	40	27	39	47
	0·3	5	—	22	27	36	61
4-Methoxycoumarin	0	82	93	95	85*	—	—
	0·003	90	88	88	86*	—	—
	0·05	31	12	32	37*	—	—

* Concentration of G.A. in this case 0·05 mg/dish

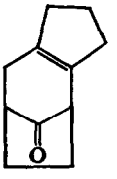
TABLE 5. (cont'd). EXTRACT FROM ANALYSIS OF VARIANCE. PROBABILITIES FOR F IN APPROPRIATE SOURCES OF VARIANCE

	3,4-Dihydrocoumarin	3,4,5,6,7,8-Hexahydrocoumarin	4-Methoxycoumarin
Replicates	N.S.	N.S.	N.S.
G.A.	$p<0·01$	$p<0·01$	N.S.
Compound	$p<0·01$	$p<0·01$	$p<0·01$
Interaction G.A. × Compound	$p<0·01$	$p<0·01$	N.S.

Several compounds not structurally related to coumarin were tested and of these activity was confined to compounds having the 3,2,1,-bridged ring system (II [active], III [inactive]) and some lactones. Both of these are structural features of the gibberellin molecule (IV). As a consequence it is postulated that coumarin may exericse its action by competing with gibberellin for a receptor site which consummates a union with the lactone group.

When the results from experiments factorially designed to test for interaction between gibberellin and some of the more active of the coumarin derivatives are analysed the following was found (ref. Tables 1 and 5). Coumarin and 4-methoxycoumarin do not interact with

gibberellin in a fashion which indicates that there is competition for an active site, while dihydro- and hexahydrocoumarin do.



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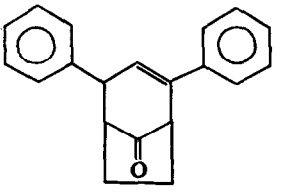

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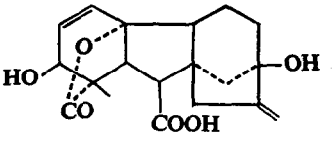

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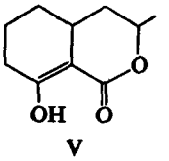
Although it has been suggested that the action of coumarin is brought about by competition for a site, this can only be demonstrated if there exists a true interaction between this compound and gibberellic acid. The inability to demonstrate interaction in the case of coumarin and 4-methoxy-coumarin possibly lies in selective metabolic destruction of coumarin or in the rate at which the active compounds reach the binding site. Some support for this hypothesis is afforded by the inactivity of coumarins which are highly substituted or carry bulky groups where steric hindrance may be expected to interfere with coupling processes. Also polar substituents may render the molecule unavailable for physiological action because of one (or more) of several processes. These include steric hindrance, facile selective bonding to another site in the cell, greatly increased metabolic destruction and inductive effects which modify the chemical reactivity of the molecule.

Only when the mobilities of the competing molecules are of the same order will it be possible for experiments of the design used here to give results which might show statistical and biological interaction.

It is unlikely that metabolic degradation of coumarin via dihydrocoumarin, as found by Kosuge and Conn[15] in *Melilotus alba* is occurring, because of the effectiveness of the dihydro compound in our system. In their work these authors demonstrated that dihydrocoumarin hydrolase brought about the hydrolysis of dihydrocoumarin, formed putatively by 3,4-dihydrogenation of coumarin, to melilotic acid. Various substituted coumarins were degraded to the analogous substituted melilotic acid with varying degrees of effectiveness. However dihydrocoumarin hydrolase was not universally present in the plants tested and we cannot assume its presence in lettuce.



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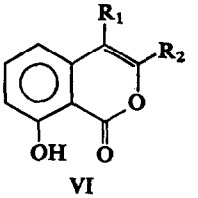

VI

MATERIALS AND METHODS

All tests were carried out using a sample of "Grand Rapids" lettuce seed obtained from The Page Seed Co. (batch No. 017563). This sample showed absolute photoblastism at 30° but none at all at 20°. When germinated at 20° in the presence of a test compound, induction of light sensitivity by the compound can be observed as follows.

[15] T. KOSUGE and E. E. CONN, *J. Biol. Chem.* **237**, 1653 (1962).

The bases of 4 cm petri dishes are lined with 2·5 cm squares of Whatman seed test filter paper 0·4 mm thick. The required amount of test substance (0·1 mg is standard) is added to the paper in 0·1 ml of suitable solvent, usually ether or methanol. As controls the paper is moistened with an equal volume of pure solvent. The solvent is allowed to evaporate. 0·5 ml of deionized water is applied to the paper and immediately 25 seeds of "Grand Rapids" lettuce, previously dispensed in small test tubes are scattered over the surface of the moist filter paper. (This gives a concentration of test solution of 200 ppm w/v as standard.) As completed, the dishes are placed in aluminium cans with screw tops. When closed these cans are light tight and the lids are screwed back half a turn to allow exchange of gases. Three replicates per dilution are employed and each test done at least twice on different occasions.

One set of three is kept in the cans while another set is exposed to light from a 20 W Warm White fluorescent lamp between 4 and 8 hr after commencement of imbibition. The amount of light given saturates the light requirements of the seed under conditions of high temperature-induced dormancy and amounts to 2 min exposure 7·5 cm from the lamp envelope.

Counts are made at 24 and 48 hr, the first one being carried out under a safelight free from morphogenetically active light. Comparison of the results obtained was carried out using a contingency Chi-squared test, and it is possible to determine if the compound induces light sensitive dormancy or depresses germination whether the seed is exposed to light or kept in darkness. The seeds are exposed to test solution during the whole period of germination.

When a substance showed biological activity at the standard concentration a concentration series was run.

In those cases where it was deemed necessary to determine if there were interaction between the test compound and gibberellin the compounds were co-applied to the seed test paper to give a factorial series of concentrations and analysis of variance was carried out on the angular transformations of the percentage germination. In trials of this type four replicates per treatment were used.

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