Phytochemistry, 1980, Vol. 19, pp. 2059–2064. © Pergamon Press Ltd. Printed in England. 0031-9422/80/1001-2059 $02.00/0

INHIBITION OF PHOTOSYNTHESIS BY STILBENOIDS

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(*Received* 31 *January* 1980)

Key Word Index—*Spinacia oleracea*; Chenopodiaceae; chloroplasts; photosynthesis; stilbenes; bibenzyls; lunularic acid; pinosylvin.

Abstract—The effects of a number of naturally occurring and synthetic stilbenes on photosynthetic functions in isolated chloroplasts of *Spinacia oleracea* were investigated. CO_2-dependent O_2 evolution and electron flow from water to methylviologen in uncoupled chloroplasts were inhibited by all the compounds tested. Uncoupling of electron transport and photophosphorylation were also observed. Electron flow from $DCPIPH_2$ to methylviologen showed the superimposed effects of uncoupling and inhibition, the overall effect depending on the concentration of the stilbene. Pinosylvin and its methyl ethers were among the most inhibitory of the compounds tested. The implications of these findings are discussed in terms of the growth regulatory and antifungal activities of stilbenoids.

INTRODUCTION*

The stilbenoids are a group of naturally occurring phenolic compounds which are biosynthesized via the phenylpropanoid/polymalonate pathway and whose structures are related to those of the hydrocarbons stilbene, bibenzyl and phenanthrene [1]. They are widely distributed in plants and appear to perform a variety of functions. High concentrations of these compounds are found in some woody tissues where they may confer resistance to fungal and insect attack. In the Chinese yam (*Dioscorea batatas*) [2–4] and in the liverwort *Lunularia cruciata* [5–8] bibenzyls are reported to be involved in dormancy responses. Although the growth inhibitory properties of stilbenoids are well-documented [5–17], their mode of action has not been established. Recent reports have shown that stilbenoids can affect IAA-oxidation [18–20], respiration [21,22], photosynthesis [22] and plasma membrane ATPase activity [23–25]. In particular, Iino *et al.* [22] have described the effects of the batatasins on the activities of isolated spinach chloroplasts and potato mitochondria. The purpose of the present work was to examine the inhibition of chloroplast functions in the presence of a range of other stilbenoids.

RESULTS AND DISCUSSION

The rates of O_2 evolution from isolated spinach chloroplasts in the presence of various stilbenoids are given in Table 1. As can be seen from the example of the effect of lunularic acid shown in Fig. 1, the rate of O_2 evolution changed with time at a rate which depended on the concentration of the stilbenoid in the reaction medium. Similar results were obtained by Iino *et al.* [22]. For this reason, measurements of O_2 evolution rates were taken at a fixed time (2 min) after the addition of the compound to the reaction medium to give a final concentration of 5×10^{-4} M. The negative values in Table 1 thus indicate O_2 absorption. Chlorophorin and piceatannol, both tetrahydroxystilbenes, were the most inhibitory of the compounds tested, followed by 3,4-dihydroxystilbene, the pinosylvins and resveratrol. Phenol and ABA were inactive, and *trans*-stilbene only slightly inhibitory. In contrast, the fully methylated 3,4-dimethoxystilbene and PDME (3,5-dimethoxystilbene) were very inhibitory. The higher plant inhibitor, ABA, was previously shown to have little effect on photosynthetic activity [26–28]. Inhibition of CO_2-dependent O_2

Table 1. Effect of stilbenoids on CO_2-dependent O_2 evolution in isolated spinach chloroplasts

Compound (5×10^{-4} M)	O_2 Evolution rate (μmol O_2/mg chl/hr)
Control	94.0
trans-Stilbene	87.4
Phenol	98.7
ABA	101.3
3-Hydroxystilbene	34.5
4-Hydroxystilbene	5.1
4,4′-Dihydroxystilbene	17.9
Stilbestrol	−12.6
Oxyresveratrol	−14.5
4-Hydroxybibenzyl	−16.7
3,4-Dimethoxystilbene	−17.2
PMME	−20.2
PDME	−21.0
Pinosylvin	−21.0
3,4-Dihydroxystilbene	−22.5
Resveratrol	−24.1
Piceatannol	−32.6
Chlorophorin	−52.8

* Abbreviations used: MV = methylviologen, $DCPIPH_2$ = dichlorophenolindophenol, PMME = pinosylvin monomethyl ether, PDME = pinosylvin dimethyl ether, LNA = lunularic acid, ABA = abscisic acid.

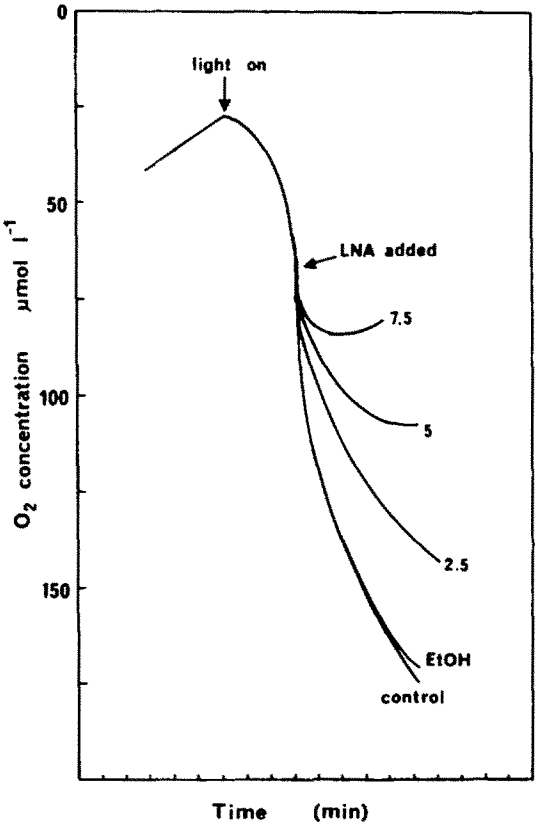

Fig. 1. Time course of inhibition of CO_2-dependent O_2 evolution in isolated spinach chloroplasts by lunularic acid (LNA) ($\times 10^{-4}$ M).

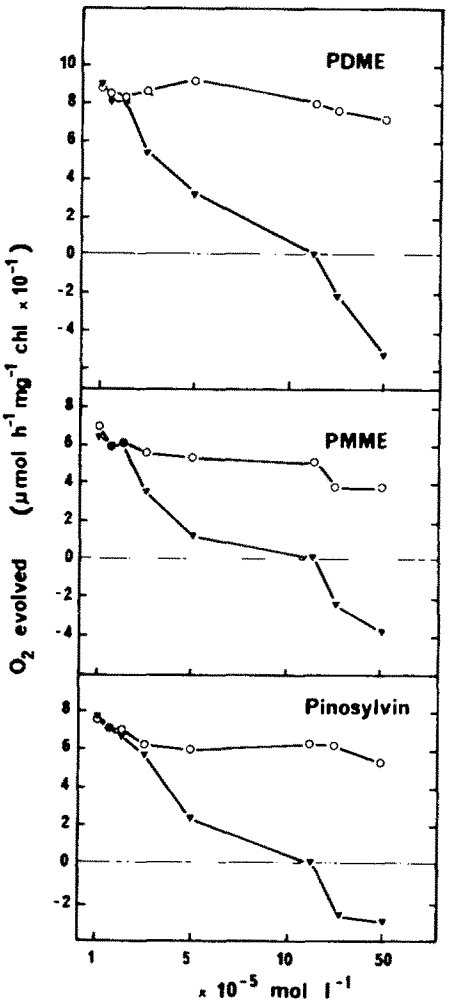

Fig. 2. Inhibition of CO_2-dependent O_2 evolution in isolated spinach chloroplasts at various concentrations of pinosylvin, pinosylvin monomethyl ether (PMME) and pinosylvin dimethyl ether (PDME). Control (○), stilbene (▼).

Table 2. Effects of stilbenoids on photoreduction of MV in uncoupled spinach chloroplasts

Compound (5×10^{-4} M)	O_2 Uptake µmol/mg chl/hr (%)
Control	789 (100)
LNA	449 (57)
Phenol	339 (43)
Piceatannol	337 (43)
Phenanthrene	261 (33)
Resveratrol	263 (33)
trans-Stilbene	246 (31)
4-Hydroxystilbene	207 (26)
3,4-Dihydroxystilbene	183 (23)
3,4-Dimethoxystilbene	165 (21)
4,4'-Dihydroxystilbene	128 (16)
Hydrangenol	111 (14)
Oxyresveratrol	55 (7)
3-Hydroxystilbene	49 (6)
PDME	39 (5)
Stilbestrol	37 (5)
Pinosylvin	34 (4)
Chlorophorin	25 (3)
4-Hydroxybibenzyl	20 (3)
PMME	11 (1)

evolution in the presence of the liverwort growth inhibitor LNA is shown in Fig. 1. At the lower concentrations tested, LNA appears to be about as effective an inhibitor of CO_2-dependent O_2 evolution as the batatasins [22], although comparison of the two sets of data is complicated by the change in rate with time.

A more detailed study of the effects of pinosylvin and its methyl ethers is shown in Fig. 2. All three compounds completely inhibited O_2 evolution at 2.5×10^{-4} M, i.e. these compounds were also at least as inhibitory as batatasin I.

The effects of stilbenoids on the photoreduction of MV by disrupted, uncoupled spinach chloroplasts are shown in Table 2. Once again the pinosylvins, together with chlorophorin, 4-hydroxybibenzyl, 3-hydroxystilbene and oxyresveratrol, are potent inhibitors, in this case of electron flow from water to MV. The pinosylvins were inhibitory at concentrations down to 1.25×10^{-5} M, the methyl ethers being the most effective (Figs. 3 and 4). PDME was also tested with chloroplasts isolated and resuspended in media in which glycinebetaine replaced the sorbitol. Glycinebetaine is considered to be an

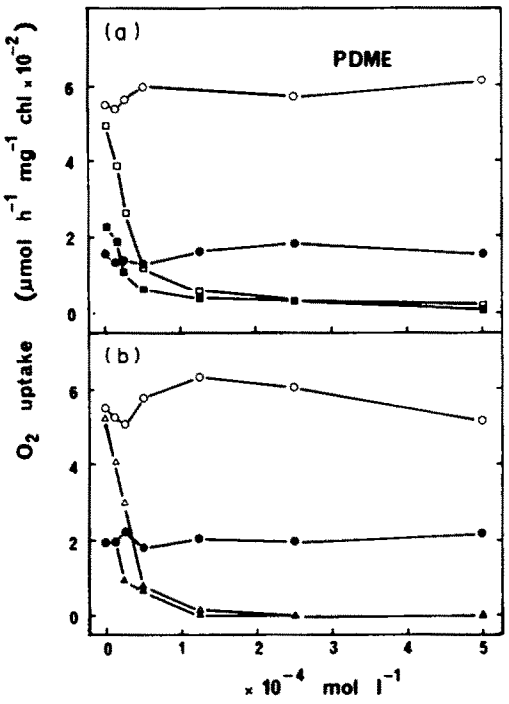

Fig. 3. Inhibition of photoreduction of MV by disrupted spinach chloroplast in the presence (closed symbols) or absence (open symbols) of the uncoupler NH_4Cl. Controls (●, □); pinosylvin or pinosylvin monomethyl ether (PMME), (▲, △).

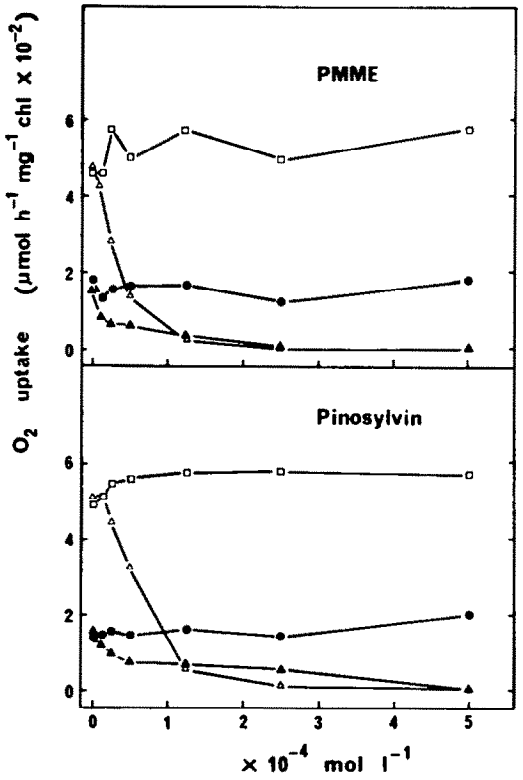

Fig. 4. Inhibition of photoreduction of MV by disrupted spinach chloroplasts in the presence (closed symbols) or absence (open symbols) of the uncoupler NH_4Cl. Controls (●, □); pinosylvin dimethyl ether (PDME), (■, □). (a) Chloroplasts isolated in glycinebetaine; (b) chloroplasts isolated in sorbitol.

important cytoplasmic osmoticum in the Chenopodiaceae and its use for the isolation of spinach chloroplasts has been described by Larkum and Wyn Jones [29]. The results obtained in the present experiments with spinach chloroplasts isolated in glycinebetaine (Fig. 4a) were similar to those obtained with chloroplasts isolated in sorbitol (Fig. 4b). The chloroplasts isolated in glycinebetaine, however, show a vestigial electron flow over the range $1.25–5.0 \times 10^{-4}$ M PDME, whilst those isolated in sorbitol have essentially no electron flow in this range of PDME concentrations. The mechanisms by which spinach chloroplasts isolated in glycinebetaine are more stable to senescence than those isolated with sorbitol as the osmoticum are still not known. It is probable that it is not a simple membrane stabilization phenomenon (Coughlan, unpublished results) and work is in progress on the protective mechanism.

Results of a more detailed study of MV reduction are presented in Table 3. In the conditions used, 4-hydroxybibenzyl, stilbestrol and PDME inhibited O_2 uptake in the absence of the uncoupler NH_4Cl. 3,4-Dimethoxystilbene and phenanthrene were clearly acting as uncouplers themselves. The addition of 1,5-diphenylcarbazide (which donates electrons to photosystem II, thus bypassing the site of H_2O splitting) to chloroplasts inhibited by stilbenoids in some cases overcame the

Table 3. Effect of 1,5-diphenylcarbazide on MV photoreduction in the presence of various stilbenoids

Compound	O_2 Uptake (μmol/mg chl/hr) (%)					
	Control		Stilbenoid (5×10^{-4} M)		Stilbenoid +1.5 mM DPC	
10 mM NH_4Cl	+	−	+	−	+	−
4-Hydroxybibenzyl	172	34	8 (5)	2 (6)	26 (15)	21 (62)
PDME	241	59	26 (11)	17 (28)	30 (12)	42 (71)
Stilbestrol	207	50	9 (4)	7 (13)	17 (8)	17 (34)
4,4'-Dihydroxystilbene	259	55	38 (15)	22 (40)	59 (23)	51 (93)
Oxyresveratrol	250	50	19 (8)	52 (103)	28 (11)	43 (86)
Chlorophorin	257	66	20 (8)	28 (42)	44 (7)	36 (55)
Phenanthrene	257	53	87 (34)	84 (158)	74 (29)	84 (158)
3,4-Dimethoxystilbene	218	42	117 (54)	131 (310)	74 (40)	84 (158)

Table 4. Effect of stilbenoids on electron flow from $DCPIPH_2$ to MV

Compound	Concentration ($\times 10^{-4}$ M)	O_2 Uptake μmoles/mg chl/hr (%)			
		Controls		Stilbenoid	
NH_4Cl	100	+	−	+	−
Phenol	12.5	724	189	831 (115)	248 (131)
trans-Stilbene	12.5	657	189	831 (126)	342 (180)
3,4-Dihydroxystilbene	12.5	804	189	938 (117)	1085 (574)
4-Hydroxystilbene	12.5	831	189	1072 (129)	743 (393)
Piceatannol	12.5	670	236	1072 (160)	307 (130)
3-Hydroxystilbene	12.5	537	259	750 (140)	354 (137)
Phenanthrene	12.5	670	230	670 (100)	637 (277)
Stilbestrol	2.5	912	218	804 (88)	802 (368)
	6.25	724	231	912 (126)	649 (281)
	12.5	643	212	940 (146)	590 (278)
Hydrangenol	2.5	791	224	912 (115)	330 (147)
	6.25	871	236	1059 (121)	925 (392)
	12.5	831	200	885 (106)	790 (395)
Chlorophorin	2.5	1019	295	885 (87)	590 (200)
	6.25	777	189	992 (128)	460 (243)
	12.5	643	200	692 (108)	436 (218)
4-Hydroxybibenzyl	2.5	671	189	770 (115)	448 (237)
	6.25	581	204	935 (161)	720 (353)
	12.5	1010	228	1200 (119)	864 (379)
Oxyresveratrol	6.25	590	212	509 (86)	177 (83)
	12.5	563	177	86 (15)	71 (40)

inhibition of electron flow to MV (e.g. in the case of 4-hydroxybibenzyl and PDME). Thus one site of action of these stilbenoids could be the water-splitting reaction. A similar restoration of the ability to reduce MV to spinach chloroplasts inhibited by batatasins was observed by Iino *et al.* [22].

It is probable that the effects of inhibition and uncoupling are superimposed. The pinosylvins (Figs. 3 and 4) were much more potent inhibitors of electron flow from H_2O to MV than the batatasins. Thus the uncoupling action seen with the batatasins [22] was not evident in the present study of the effects of the pinosylvins where inhibition was almost complete at 1×10^{-4} M.

Table 4 lists the rates of O_2 uptake in a reaction mixture designed to measure electron flow from $DCPIPH_2$ to MV. As with the batatasins, many stilbenoids showed a greater stimulation of O_2 uptake in the absence of NH_4Cl, suggesting that part of the action of these compounds is to uncouple electron transport in photosystem I from photophosphorylation. Oxyresveratrol inhibited O_2 uptake at concentrations of 1.25×10^{-3} and 6.26×10^{-4} M. As in the case of electron transport from H_2O to MV, the presence of the stilbenoids resulted in both inhibition of electron transport and uncoupling, depending on the concentration of the stilbenoid. This can be seen in the results of the more detailed experiment shown in Table 5. At low concentrations ($< 1 \times 10^{-4}$ M), PMME acted, at least partly, as an uncoupler. At concentrations above 2.5×10^{-4} M, inhibition of O_2 uptake, and hence of electron flow, was observed.

The results of experiments with notholaenic acid (3-hydroxy-5,4′-dimethoxybibenzyl-2-carboxylic acid), recently identified as a component of the farina of ferns belonging to the genus *Notholaena* [30], are shown in Table 6. It appears to be unable to penetrate the outer chloroplast envelope as evidenced by the fact that CO_2-dependent O_2 evolution was relatively unaffected by 5×10^{-4} M notholaenic acid. Coupled electron flow from H_2O to MV was relatively unaffected by this concentration of notholaenic acid, but electron flow uncoupled from photophosphorylation by 10 mM NH_4Cl was completely

Table 5. The effects of PMME (3-hydroxy-5-methoxystilbene) on electron flow from $DCPIPH_2$ to MV in coupled and uncoupled spinach chloroplasts

PMME concentration (M)	Oxygen uptake rate (% of controls)	
	$-NH_4Cl$	$+NH_4Cl$
Expt. (a) ($\times 10^{-5}$)		
0	100	316
2.5	183	279
5.0	226	289
7.5	261	280
10.0	294	275
12.5	292	281
Expt. (b) ($\times 10^{-4}$)		
0	100	262
2.5	273	277
5.0	190	191
7.5	157	161
12.5	58	106

Table 6. Effects of notholaenic acid on chloroplast activities

	NH_4Cl	Control	Notholaenic acid (5×10^{-4} M)
CO_2-dependent O_2 evolution (%)	–	100	87
MV photoreduction (μmol O_2 uptake /mg chl/hr)	–	212	180
	+	551	0
Electron flow from $DCPIPH_2$ to MV (μmol O_2 uptake /mg chl/hr)	–	347	1200
	+	1200	1200

inhibited. Photosystem I electron flow was completely uncoupled by notholaenic acid.

The results presented above show that a large number of stilbenoids have the ability to modify chloroplast activities. Inhibition of CO_2-dependent O_2 evolution and electron transport from H_2O to MV and (at higher concentrations) from $DCPIPH_2$ to MV were observed. At lower concentrations photoreduction of MV in the presence of $DCPIPH_2$ was stimulated by the stilbenoids, particularly in the absence of the uncoupling agent NH_4Cl. Part of the effect of the stilbenoids appears to involve the uncoupling of electron transport from photophosphorylation. In addition to the relative uncoupling and inhibitory activities of the stilbenoids, their solubilities in the aqueous phase and in the chloroplast membranes may influence their effectiveness.

Many of the stilbenoids tested here showed activities comparable to those of the batatasins investigated earlier [22]. In particular, the pinosylvins were found to be potent inhibitors of photosynthesis at all three levels of methylation. These compounds belong to the large group of stilbenoids which accumulate, often to high levels, in the heartwood of many woody species. Their effect on photosynthesis (and probably on respiration [21,22] may account for their exclusion from metabolically active tissue. At the same time, the antifungal activities of the pinosylvins may be due, in part, to similar inhibitory effects on fungal metabolism.

Another of the compounds tested above, namely resveratrol, has recently been implicated in some phytoalexin responses. In *Vitis vinifera* resveratrol and its oligomers accumulate when leaves are infected with pathogens or exposed to UV radiation [31–34], whilst in *Arachis hypogaea* resveratrol and the 4-isopentenyl derivative are formed [35,36]. CO_2-dependent O_2 evolution in isolated spinach chloroplasts was completely inhibited by resveratrol at 5×10^{-4} M (Table 1). Thus the production of such antifungal compounds in response to infection of the leaf may occur at the expense of normal metabolic activities. Most stilbenoids are not normally major constituents of green tissues. However, the liverwort growth inhibitor LNA occurs in the green tissues of some thallose liverworts at concentrations (up to 600 μg/g fr. wt) which would completely inhibit photosynthesis and growth if applied externally [9]. The intracellular location of LNA in liverworts remains unclear, but the present results support the suggestion that it is not all free in the cytosol. As in the case of the batatasins [22], it is tempting to suggest that the growth regulatory properties of LNA may be due to its effect on photosynthesis (and possibly respiration). However, such a suggestion involves the extrapolation of results obtained with one organism to other unrelated plants.

EXPERIMENTAL

Chloroplast preparations were made from freshly harvested leaves of spinach (Arthur Yates hybrid 102) using the method of ref. [37]. Jensen and Bassham's soln was used for resuspension of the chloroplast pellet and in the reaction medium [38]. In one instance, glycinebetaine replaced sorbitol as the osmoticum in both the isolation and resuspension media. O_2 concns were measured polarographically using a Clarke-type electrode in a system similar to that described in ref. [39]. CO_2-dependent O_2 evolution, photoreduction of MV and electron flow from $DCPIPH_2$ to MV were measured as described in ref. [22]. Chlorophyll concns were estimated from Arnon's formulae [40].

The stilbenoids used in this investigation were synthesized or otherwise obtained from the sources described previously [9]. They were added to the reaction medium in ethanolic soln and similar quantities of EtOH were added to the corresponding controls. The final EtOH concn was less than that reported to inhibit photosynthesis in spinach chloroplasts [41].

Acknowledgements—We are grateful to Dr. R. G. Wyn Jones for research facilities and useful discussion, to Dr. J. Davenport for the use of oxygen electrodes, and to N. Smith, who participated in some of the experiments.

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