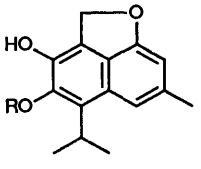
The Role of Free Radicals in the Decomposition of the Phytoalexin Desoxyhemigossypol

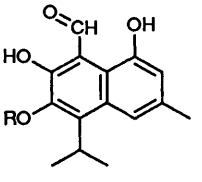
Robert D. Stipanovic, Marshall E. Mace, Alois A. Bell and Ross C. Beier
U.S. Department of Agriculture, Agricultural Research Service, Southern Crops and Food Animal Protection Research Laboratories, Route 5, Box 805, College Station, TX 77845, USA

The cotton phytoalexin desoxyhemigossypol (dHG) decomposed rapidly in solution to give hemigossypol (HG). The rate of decomposition was retarded by the reducing agents ascorbic acid, reduced glutathione and cysteine, by the metal chelator diethylenetriaminepentaacetic acid, and by the enzyme catalase. However, the chelator, ethylenediaminetetraacetic acid did not reduce the rate of decomposition and the enzyme superoxide dismutase increased the rate of decomposition. Solutions of the phytoalexin desoxyhemigossypol-6-methyl ether were significantly more stable than were those of dHG. Oxygen-18 from water but not from oxygen gas was incorporated into HG during this decomposition. A hydroperoxynaphthalenone which loses hydrogen peroxide is proposed as an intermediate to explain this observation. The formation of hydrogen peroxide may be involved in the toxicity of this phytoalexin to plant pathogens such as *Verticillium dahliae*.

In response to infection by a pathogen, plants produce secondary metabolites called phytoalexins which are toxic to the pathogen. The cotton plant *Gossypium hirsutum* produces four phytoalexins **1a,b** and **2a,b** in response to infection by the wilt pathogen *Verticillium dahliae*. Current evidence indicates



1a R = H (dHG)
1b R = Me (dMHG)



2a R = H (HG)
2b R = Me (MHG)

desoxyhemigossypol (dHG) **1a** is the most important of these four phytoalexins because it is the most toxic to *V. dahliae* and the most water soluble at pH 6.3, the pH of infected xylem of cotton stems.[1] It required 15 μg cm^{-3} of dHG to kill all *V. dahliae* mycelia, whereas its methyl derivative (dMHG) **1b**, which is the second most toxic to *V. dahliae*, requires 25 μg cm^{-3} to achieve this effect.[1] The other phytoalexins hemigossypol (HG) **2a** and hemigossypol-6-methyl ether (MHG) **2b** require 30 μg cm^{-3} and 50 μg cm^{-3}, respectively.

Of the four cotton phytoalexins dHG is also the most labile. It was found to decompose rapidly in solution to give HG. Its methylated derivative, dMHG, was significantly more stable. Since dHG lacks the aldehyde functional group found in HG, which is often associated with biological activity, we hypothesized the mechanism of decomposition could be related to its toxicity. Thus, we began a systematic investigation of the decomposition of dHG. Since dHG is of interest because of its phytoalexin activity, the solutions selected to study this decomposition were designed to closely mimic the media used to investigate its toxicity to *V. dahliae*. For example, buffered deionized aqueous solutions with and without glucose and/or trace elements normally used in *V. dahliae* growth media[2] were used in most of these studies.

Results and Discussion

In pH 6.3 phosphate buffer solution, dHG rapidly decomposed. This decomposition was significantly slower in the pH 6.3 buffered media which contained glucose (Table 1). Similarly, when solutions were degassed with argon, the rate of decomposition was mitigated. Trace elements in the media increased the rate of decomposition. The product of decomposition was HG (64% yield).

Addition of the reducing agents, ascorbic acid, reduced glutathione and cysteine significantly reduced the rate of decomposition (Table 1). The metal chelator, diethylenetriaminepentaacetic acid (DTPA), reduced the rate of decomposition, but ethylenediaminetetraacetic acid (EDTA) had the opposite effect (Table 1). The enzyme catalase reduced the rate of decomposition of dHG, while superoxide dismutase (SOD) increased the decomposition rate.

The reduction in rate of decomposition under an atmosphere of argon, and the increase in rate of decomposition when transition metals are added (Table 1) both implicate a free radical oxidation as the operative mechanism of decomposition of dHG to form HG. The reduction in the rate of decomposition in the glucose media *vs.* buffer alone is also in accord with this hypothesis. For example, the conversion of $O_2^{\bullet -}$ to $HO^{\bullet}$ in the presence of Fe^{3+} was reduced ~50% by glucose (40 μmol dm^{-3}),[3] and fructose and inositol exhibited similar effects. Glucose can act as a hydrogen atom donor in free radical reactions.[4] Furthermore, the reducing agents, ascorbic acid, reduced glutathione and cysteine retard the rate of decomposition (Table 1). These results indicate dHG decomposes by a free radical process but provide only scant evidence as to the initiator of the free radical sequence.

Enzymes that utilize specific substrates can provide evidence on the exact character of this initiator. Thus, superoxide ($O_2^{\bullet -}$) is the specific substrate for the enzyme SOD. The product of dismutation is hydrogen peroxide (H_2O_2) (Reaction 1). SOD (1700 U cm^{-3}) increased the rate of decomposition of dHG (Table 1). However, catalase (1700 U cm^{-3}), which destroys H_2O_2 (Reaction 2), effectively stabilized dHG in solution.

$$2O_2^{\bullet -} + 2H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2 \quad (1)$$

$$H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2 \quad (2)$$

The decomposition of dMHG in pH 6.3 phosphate buffer and pH 6.3 buffered media was significantly slower than dHG. Even after 72 hours only 8 and 10% of the dMHG decomposed in

Table 1 Decomposition of desoxyhemigossypol (dHG) in various media

Media	dHG (%) After Time Elapsed	
	24 h	48 h
Buffer[a]	42	13
Buffered Medium[b]	63	31
Buffered Medium under Argon	75	55
Buffered Medium + Trace Elements[c]	42	10
Buffered Medium + Ascorbic Acid (1 mmol dm^{-3})	93	88
Buffered Medium + Cysteine (0.5 mmol dm^{-3})	83	59
Buffered Medium + Reduced Glutathione (1 mmol dm^{-3})	86	49
Buffered Medium + Diethylenetriaminepentaacetic Acid (1 mmol dm^{-3})	78	46
Buffered Medium + Ethylenediaminetetraacetic Acid (1 mmol dm^{-3})	56	22
Buffered Medium + Superoxide Dismutase (1700 U cm^{-3})	42	12
Buffered Medium + Catalase (1700 U cm^{-3})	82	65

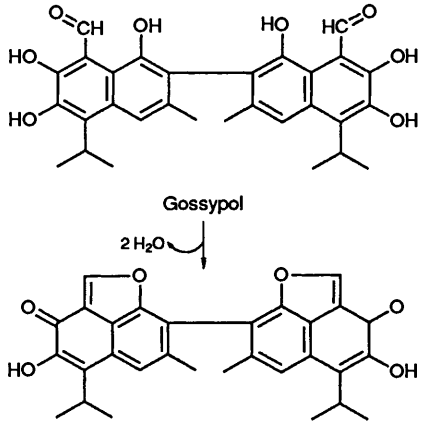
[a] Phosphate Buffer, 150 mmol dm^{-3}, pH 6.3 in dimethylsulfoxide (20 mm^3) and deionized water (1 cm^3). [b] Buffered as above plus glucose (125 mmol dm^{-3}), $MgSO_4$ (1 mmol dm^{-3}), $NH_4H_2PO_4$ (30 mmol dm^{-3}). [c] Trace elements as described by Esposito and Fletcher, 1961.[2]

Table 2 The ratios of ions formed in the mass spectrum of hemigossypol formed by the decomposition of desoxyhemigossypol in $^{18}O_2$, $H_2{}^{18}O$ and of a standard

Ratios of ions (*m/z*)	Standard	$^{18}O_2$	$H_2{}^{18}O$
262/260	4	5	22
244/242	8	9	6

buffer and media, respectively. This implicates the importance of the hydroxy group ortho to the isopropyl moiety in facilitating the decomposition of dHG.

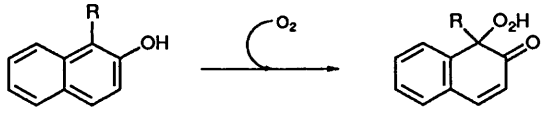
Electron spin resonance experiments failed to detect any long lived radicals. This lead to a study of the mass spectrum of HG formed from dHG in the presence of oxygen-18. Surprisingly, the *m/z* 262 and 260 ratio of HG formed under an $^{18}O_2$ atmosphere (50% $^{18}O_2$) was not significantly different from a standard (Table 2). However, when dHG was allowed to decompose in $H_2{}^{18}O$ (50% oxygen-18), a significant increase in this ratio was noted (Table 2). Gossypol (the dimer of HG) is known to readily lose H_2O both chemically[5,6] and in the mass spectrometer[7] as shown in Scheme 1.[8] Hemigossypol



Scheme 1

undergoes a similar dehydration in the mass spectrometer.[9] The oxygen lost in this dehydration is from the aldehyde group. Thus, one expects and indeed observes the *m/z* 244 and 242 ratios in HG derived from the decomposition of dHG in $H_2{}^{18}O$ and that of a standard do not differ significantly (Table 2).

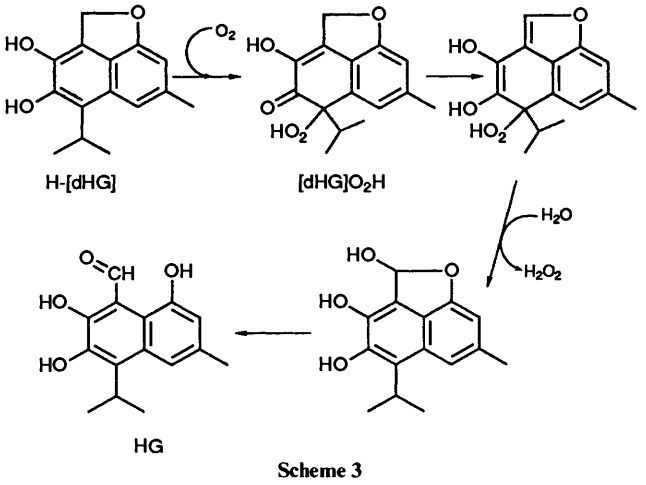
Carnduff and co-workers[10–12] reported that 1-isopropyl-2-naphthol and several related 1-alkyl-2-naphthols are converted on standing in air as a solid or in solution into hydroperoxynaphthalenones (Scheme 2). Thus, 1-isopropyl-2-naphthol is



Scheme 2

readily converted into the crystalline 1-hydroxynaphthalenone in 75% yield.[10] Subsequently, Carnduff showed that increased strain due to congestion between the peri-substituents correlates with an increase in the rate of formation of the alkylhydroperoxide.[11] Carnduff also showed that a series of aryl- and aralkyl-naphthols (*i.e.* R = Ph, CH_2Ph, $CHPh_2$, $CMePh_2$ and CPh_3 in Scheme 2) failed to react with oxygen.[13] He ascribed this stability to hydrogen bonding between the phenol and the aryl groups, which prevents hydrogen atom abstraction from the OH group.

A similar reaction appears to be involved in the oxidation of dHG as proposed in Scheme 3. One expects the initial oxidation



Scheme 3

to form the hydroperoxynaphthalenone *via* a superoxide ($O_2^{\bullet -}$) driven reaction (Reactions 3–7) which is derived from trace

$$O_2 + Fe^{2+} \rightleftharpoons O_2^{\bullet -} + Fe^{3+} \quad (3)$$

$$O_2^{\bullet -} + H^+ \longrightarrow HO_2^{\bullet} \quad (4)$$

$$HO_2^{\bullet} + O_2^{\bullet -} + H^+ \longrightarrow H_2O_2 + O_2 \quad (5)$$

$$2HO_2^{\bullet} \longrightarrow H_2O_2 + O_2 \quad (6)$$

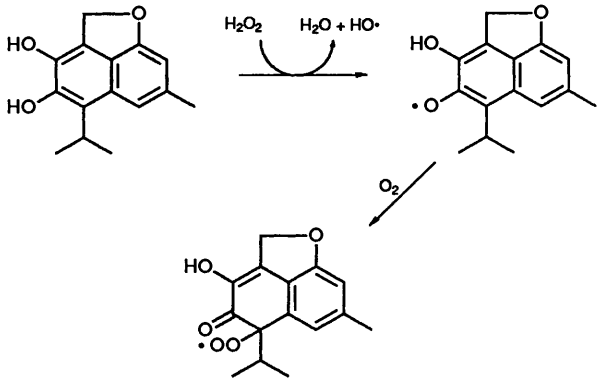
$$H_2O_2 + Fe^{2+} \longrightarrow HO^{\bullet} + OH^- + Fe^{3+} \quad (7)$$

quantities of reduced transition metals in solution. DTPA has been shown to decrease the rate of such a reaction[14] by blocking the reduction of Fe^{3+} by $O_2^{\bullet -}$;[15] EDTA was shown to increase the rate of a $O_2^{\bullet -}$ driven Fenton reaction.[16] Storing the solution under an inert atmosphere or the addition of reducing agents would be expected to retard the formation of the hydroperoxynaphthalenone. SOD dismutates $O_2^{\bullet -}$ (Reaction 1), which is involved in the Fenton reaction sequence. The enzyme product from Reaction 1 is H_2O_2, which provides a mechanism for increasing the rate of formation of the hydroperoxynaphthalenone. However, catalase destroys H_2O_2 (Reaction 2), and thus interferes with the Fenton reaction sequence,[16] and destroys the H_2O_2 produced in Scheme 3 and in Reaction 8, the dismutation of $O_2^{\bullet -}$ in aqueous media. The

$$2O_2^{\bullet -} + 2H^+ \rightleftharpoons H_2O_2 + O_2 \quad (8)$$

equilibrium for this latter reaction even at neutral pH is far to the right (K_{pH7} 4 × 10^{20}).[17]

Carnduff's work on the aryl- and aralkyl-naphthols[13] indicates the initial step in the oxidation is the formation of the phenoxy radical. Thus, we would propose Scheme 4 as a



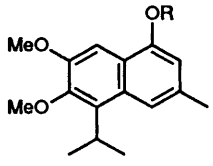
Scheme 4

plausible route to form the hydroperoxide in which H_2O_2 formed *via* the Fenton reaction, abstracts a hydrogen atom from the phenol group and provides a reactive site to which O_2 can add. This shows the importance of a phenol group *ortho* to the isopropyl unit in facilitating the decomposition. This also explains why dMHG, which lacks a free phenol group at this position is significantly more stable than dHG.

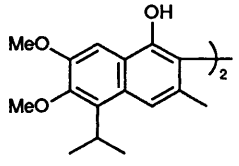
Conclusions.—The proposed mechanism (Scheme 3) for the decomposition of dHG agrees with the experimental observations including the stabilizing effect of catalase, the incorporation of oxygen-18 from $H_2{}^{18}O$ and the greater stability of dMHG *vs.* dHG. The production of H_2O_2 as a by-product of this reaction (Scheme 3) could have important implications in the defence response in plants. For example, the production of H_2O_2[18] and $O_2^{\bullet -}$ (ref. 19) in response to a pathogen-derived elicitor has been confirmed in soybean cell suspension cultures, and tobacco leaf discs infected with tobacco mosaic virus showed a marked increase in $O_2^{\bullet -}$ generating activity.[20]

The autoxidation of dHG as proposed in Scheme 3 does not require light to initiate this free radical process. This is consistent with the location of dHG within the stem and root, where it is protected from light.[1] A light-induced mechanism would be consistent with phytoalexins produced in foliar plant parts. 2,7-Dihydroxycadalene, the phytoalexin produced in cotton leaves, is a likely candidate to operate by this mechanism.[21–23] A free radical oxidation has been implicated in the decomposition of the elm tree metabolite 7-hydroxycadalene in air on silica gel plates.[24]

Oxygen-free radicals apparently also are involved in the spermicidal activity of the HG dimer, gossypol, which is being widely studied as a male antifertility agent. For example, in the presence but not in the absence of SOD or catalase, gossypol-treated sperm was found to fertilize sea urchin eggs normally.[25] Gossypol promoted both the formation of $O_2^{\bullet -}$ in rat liver microsomes and a radical species that was partially quenched by SOD, but was more effectively quenched by catalase in human sperm cell suspensions.[26] Studies have shown that gossypol disrupts membrane order, permeability and hexose transport.[27] Gossypol increased isolated islet cell membrane permeability as indicated by Trypan Blue uptake,[28] and Trypan Blue uptake was inhibited by SOD, catalase, and DTPA.



3a R = H
3b R = Me



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Furthermore, compound **3a** exhibits spermicidal activity equal to gossypol,[29] but the trimethyl ether derivative **3b** is inactive. Compound **3a** readily dimerizes to form compound **4**, while **3b** does not dimerize. This dimerization is presumably catalysed by oxygen.[30]

Experiments to be reported elsewhere show the compounds and enzyme that protect dHG from decomposition generally reduce the toxicity of dHG *in vitro* to *V. dahliae.*

Experimental

Preparation of Desoxyhemigossypol (dHG) **1a**.—Desoxyhemigossypol-6-methyl ether (dMHG) (29 mg), isolated from infected cotton stems,[31] was dissolved, under a nitrogen atmosphere and anhydrous conditions, in anhydrous 1,2-dichloroethane (4 cm^3). This solution was added slowly to a flask containing boron tribromide–dimethyl sulfide complex (107 mg) and 1,2-dichloroethane (10 cm^3) under anhydrous conditions. The mixture was stirred under nitrogen at room temperature for 5 h. Water (10 cm^3) was added to the flask followed by a saturated brine solution (10 cm^3). The aqueous solution was extracted with ether (3 × 25 cm^3). The ether layer was washed with 6 mol dm^{-3} HCl (1 × 25 cm^3) and with saturated brine (3 × 25 cm^3). The organic solution was dried (Na_2SO_4) and evaporated to dryness. The residue was chromatographed over silica gel (30 g, J. T. Baker), eluting with hexane–ether, 1:0 (60 cm^3), 2:3 (100 cm^3) and 1:1 (100 cm^3). The dHG elutes after a yellow band with hexane–ether (1:1) providing 11.4 mg of dHG after recrystallization from ether–hexane (m.p. 145–147 °C).

Quantitation of dHG, HG, dMHG and MHG.—The terpenoids were quantified using high performance liquid chromatography (HPLC) (Hewlett–Packard 1090 equipped with an autoinjector and diode array detector) employing a C-8 column (Scientific Glass Engineering, 5μ, 4 × 250 mm) and a linear gradient of H_2O–MeOH–H_3PO_4 beginning at 80:20:0.07 and progressing to 40:60:0.07 in 5 min and ending at 13:87:0.07 at 22 min at a flow rate of 1.25 cm^3 min^{-1}. The eluent

was monitored at 235 nm with a bandwidth of 20 nm and a reference wavelength of 550 nm with a bandwidth of 100 nm. Column temperature was 40 °C. Standard solutions of each phytoalexin were prepared; from these solutions, standard curves were calculated using linear regression analysis.

Preparation of samples. All dHG or dMHG samples were weighed individually into standard 2 cm^3 autosampler vials. Argon was allowed to gently flow into and thus displace the air in those vials run under this inert gas. All aqueous solutions were prepared using deionized water. Phosphate buffer (150 mmol dm^{-3}, pH 6.3) or phosphate buffered medium (pH 6.3) containing glucose (125 mmol dm^{-3}), $MgSO_4$ (1 mmol dm^{-3}) and $NH_4H_2PO_4$ (30 mmol dm^{-3}) was prepared. The dHG (0.1 mg) was dissolved in dimethyl sulfoxide (DMSO) (18 mm^3), and either pH 6.3 buffer or pH 6.3 buffered medium (900 mm^3) was added. The dMHG was dissolved in DMSO (0.1 mg/214 mm^3) and pH 6.3 buffer (0.1 mg/428 mm^3) or pH 6.3 buffered media (0.1 mg/428 mm^3) was added. Samples were immediately subjected to HPLC analysis (Time = 0). Subsequent injections were taken from the same vial after various time intervals. Vials were held in a dark holder recessed in the chromatograph and thus protected from light. All reducing agents and chelators were prepared in the pH 6.3 media as either 0.5 mmol dm^{-3} or 1 mmol dm^{-3} solutions. The enzymes, superoxide dismutase (from bovine erythrocytes) (SOD) (1700 U cm^{-3}) and catalase (from bovine liver) (1700 U cm^{-3}), were dissolved in the pH 6.3 media at the concentration indicated. The solution of chemicals or enzymes was substituted for the pH 6.3 media in diluting the DMSO solutions of dHG. An aqueous solution containing trace elements was prepared as previously described.[2] This solution contained $Na_2B_4O_7$ (2.3 μmol), $FeCl_3$ (17.8 μmol), $(NH_4)_6Mo_7O_{24}$ (0.1 μmol), $MnSO_4$ (2.1 μmol), $Cu(NO_3)_2$ (1.6 μmol) and $ZnSO_4$ (15.4 μmol). The $^{18}O_2$ incorporation experiment was performed by dissolving dHG (0.1 mg) in methyl acetate (250 mm^3); water (840 mm^3) was added and $^{18}O_2$ (50% oxygen-18) was bubbled through the solution. The vial was immediately capped, and the decomposition was followed as indicated above. The $H_2{}^{18}O$ incorporation experiment was performed by dissolving dHG (0.2 mg) in methyl acetate (1% H_2O) (350 mm^3); water (400 mm^3, 50% oxygen-18) was added. The vial was capped and analysed as above. A standard solution of HG (0.1 mg/350 mm^3 methyl acetate and 400 mm^3 water) was also prepared. All samples were evaporated to dryness and transferred to capillary melting point tubes for analysis in the mass spectrometer *via* probe. Spectra were recorded in a repetitive scan mode on a VG 70–250 EHF spectrometer using electron impact (70 eV), and a source block temperature of 190 °C. The mass spectrum of the standard agreed with published data.[9] Table 2 reports the average intensity of each peak (m/z) over seventeen consecutive spectra with a good signal to noise ratio.

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References

1 M. E. Mace, R. D. Stipanovic and A. A. Bell, *Physiol. Plant Pathol.*, 1985, **26**, 209.
2 R. Esposito and A. M. Fletcher, *Arch. Biochem.* Biophys., 1961, **93**, 369.
3 M. S. Baker and J. M. Gebicki, *Arch. Biochem. Biophys.*, 1986, **246**, 581.
4 M. S. Kharasch and A. Fono, W. Nudenberg, *J. Org. Chem.*, 1950, **15**, 763.
5 F. E. Carruth, *J. Am. Chem. Soc.*, 1918, **40**, 647.
6 R. F. Miller and R. Adams, *J. Am. Chem. Soc.*, 1937, **59**, 1736.
7 R. D. Stipanovic, A. A. Bell, M. E. Mace and C. R. Howell, *Phytochemistry*, 1975, **14**, 1077.
8 R. Adams, T. A. Geissman and J. D. Edwards, *Chem. Rev.*, 1960, **60**, 555.
9 A. A. Bell, R. D. Stipanovic, C. R. Howell and P. A. Fryxell, *Phytochemistry*, 1975, **14**, 225.
10 J. Carnduff and D. G. Leppard, *J. Chem. Soc., Chem. Commun.*, 1967, 829.
11 P. A. Brady and J. Carnduff, *J. Chem. Soc., Chem. Commun.*, 1974, 816.
12 J. Carnduff and D. G. Leppard, *J. Chem. Soc., Perkin Trans. 1*, 1976, 2570.
13 P. A. Brady, J. Carnduff and F. Monoghon, *Tetrahedron Lett.*, 1977, **1977**, 3295.
14 S. Marklund and G. Marklund, *Eur. J. Biochem.*, 1974, **47**, 469.
15 G. Cohen and P. Sinet, *FEBS Lett.*, 1982, **138**, 258.
16 M. S. Baker and J. M. Gebicki, *Arch. Biochem. Biophys.*, 1984, **234**, 258.
17 D. T. Sawyer and M. J. Gibian, *Tetrahedron*, 1979, **35**, 1471.
18 I. Apostol, P. F. Heinstein and P. S. Low, *Plant Physiol.*, 1989, **90**, 109.
19 W. A. Lindner, C. Hoffman and H. Grisebach, *Phytochemistry*, 1988, **27**, 2501.
20 N. Doke and Y. Ohashi, *Physiol. Mol. Plant Pathol.*, 1988, **32**, 163.
21 M. Essenberg, M. d'A. Dokerty, B. K. Hamilton, V. T. Henning, E. C. Cover, S. J. McFaul and W. M. Johnson, *Phytopathology*, 1982, **72**, 1349.
22 M. Essenberg, M. Pierce, J. L. Shevell, T. J. Sun and P. E. Richardson, in *Plant Cell/Cell Interactions, Current Communications in Molecular Biology*, eds. I. Susex, A. Ellingboe, M. Crouch and R. Malmberg, Cold Spring Harbor Laboratory, 1985, pp. 145–149.
23 T. J. Sun, U. Melcher and M. Essenberg, *Physiol. Mol. Plant Pathol.*, 1988, **33**, 115.
24 G. Strunz, C. M. Yu and A. Salonius, *Phytochemistry*, 1989, **28**, 2861.
25 M. Coburn, P. Sinsheimer, S. Sheldon, M. Burgos and W. Troll, *Biol. Bull.*, 1980, **159**, 468.
26 A. de Peyster, A. Quintanilha, L. Packer and M. T. Smith, *Biochem. Biophys. Res. Commun.*, 1984, **118**, 573.
27 A. de Peyster, P. A. Hyslop, C. E. Kuhn and R. D. Sauerheber, *Biochem. Pharmacol.*, 1986, **35**, 3292.
28 K. Grankvist, *Int. J. Biochem.*, 1989, **21**, 853.
29 A. Manmade, P. Herlihy, J. Quick, R. P. Duffley, M. Burgos and A. P. Hoffler, *Experientia*, 1983, **39**, 1276.
30 J. D. Edwards and J. L. Chashaw, *J. Am. Chem. Soc.*, 1957, **79**, 2283.
31 R. D. Stipanovic, A. A. Bell and C. R. Howell, *Phytochemistry*, 1975, **14**, 1809.

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