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POLYAMINES AS RADICAL SCAVENGERS AND PROTECTANTS AGAINST OZONE DAMAGE

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Abstract—Leaf injury of the ozone-sensitive tobacco cultivar Bel W 3 caused by ozone treatments was prevented to a large extent by root application of putrescine, spermidine or spermine. The titres of soluble free and conjugated putrescine and spermidine were concomitantly increased two- to three-fold after putrescine or spermidine application. The amounts of putrescine and spermidine associated with cell wall or membrane pellet fractions were elevated four to six times above levels of control plants. In order to establish whether the protective effect of polyamines against ozone damage may be caused by their proposed radical scavenging properties, the reactivities of polyamines and putrescine conjugates towards hydroxyl, *tert*-butoxyl, sulphite radicals and superoxide anions were determined Free polyamines showed relatively low rate constants with all types of radicals. Only putrescine conjugates with the effective radical scavengers caffeic, ferulic and *p*-coumaric acid had consistently high rate constants. It is concluded that scavenging of radicals by free polyamines cannot explain the protection against ozone damage observed after exogenous application.

INTRODUCTION

Polyamines are present in cells in up to millimolar amounts and are known to be important for DNA replication, cell differentiation and growth regulation [1, 2]. In plants, it is mainly putrescine biosynthesis that has been reported to respond to stress conditions such as low external pH, mineral deficiencies or exposure to sulphur dioxide or cadmium [2, 3]. A close interrelationship between polyamines and oxidative stress was documented by the finding that leaf necrosis caused by ozone in tomato plants could be suppressed by an exogenous supply of polyamines [4].

Ozone damage is generally thought to result from radical reactions [5]. Plant cells are normally protected against oxidative stress exerted by ozone-derived radicals by cellular radical scavenging systems involving α-tocopherol, glutathione or superoxide dismutase [6]. The protection afforded by polyamines against ozone damage has equally been proposed to involve scavenging of ozone-derived radicals [4]. This proposal was mainly based on the previously reported radical-scavenging properties of polyamines [7]. On the other hand, it has long been established that aliphatic amines react only slowly with hydroxyl radicals [8], rendering the postulated radical-scavenging properties of polyamines [4, 7] rather questionable. In view of the importance of polyamines in plant developmental and stress adaptation processes, their protective effects against ozone damage [4] and their radical-scavenging properties [7] have been re-examined. The effect of exogenous application of polyamines has been studied using the ozone-sensitive biomonitor plant tobacco Bel W 3. In addition, the rate constants of free polyamines and of hydroxycinnamoyl conjugates of putrescine with different types of radicals were determined. The latter were generated by specific radiation chemical and photochemical methods.

RESULTS

In vivo *studies*

Ozone (0.15 ppm, 5 hr) induced severe necrosis on recently mature leaves of the tobacco cultivar Bel W 3. Injury was efficiently reduced by root application of polyamines to the tobacco plants. Putrescine and spermidine were similarly effective (20% leaf injury at 10^{-4} M compared with 50% in untreated control plants), while spermine reduced leaf injury to 35% (at 10^{-4} M) or 15% (at 10^{-3} M) (Fig. 1).

To avoid direct effects on stomata, the polyamines were applied to the nutrient solution of tobacco plants. Root uptake of polyamines led to changes of their titres in the tobacco leaves. The levels of soluble and conjugated polyamines in control and polyamine-treated plants are shown in Fig. 2. Putrescine supply for two days (+Put; Fig. 2) increased putrescine and spermidine levels by 50–100%. Spermidine application resulted in a three-fold increase in the level of this polyamine, and putrescine was also elevated by this treatment. Both free and conjugated putrescine and spermidine levels were increased. Conjugated polyamines were 50–70% of the total soluble polyamines and conjugated putrescine was mainly found as mono-caffeoyl putrescine as determined by thin-layer co-chromatography with reference compounds (data not shown). Spermine supply did not influence the levels of putrescine and spermidine, and spermine titres were only slightly enhanced.

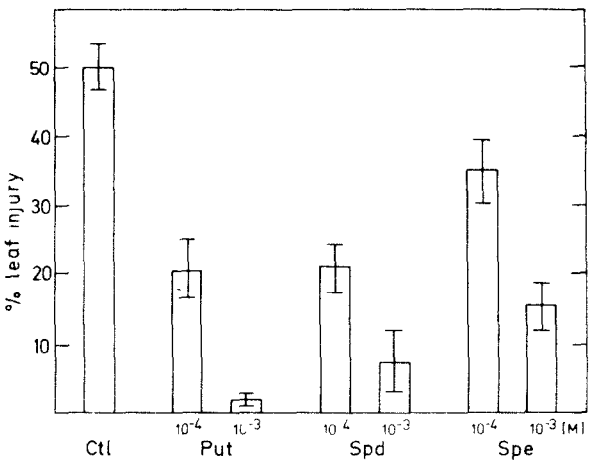

Fig 1 Effect of polyamine supply on ozone-induced leaf injury of tobacco Bel W 3 plants Polyamines were added to the nutrient solutions of hydroponically grown tobacco plants for two days Plants were then fumigated with ozone (0 15 ppm, 5 hr) and the percentage of leaf injury of leaves three and four was rated after 48 hr of further culture Bars represent ± s e of three replicates Abbreviation Ctl = control, Put = putrescine, Spd = spermidine, Spe = spermine

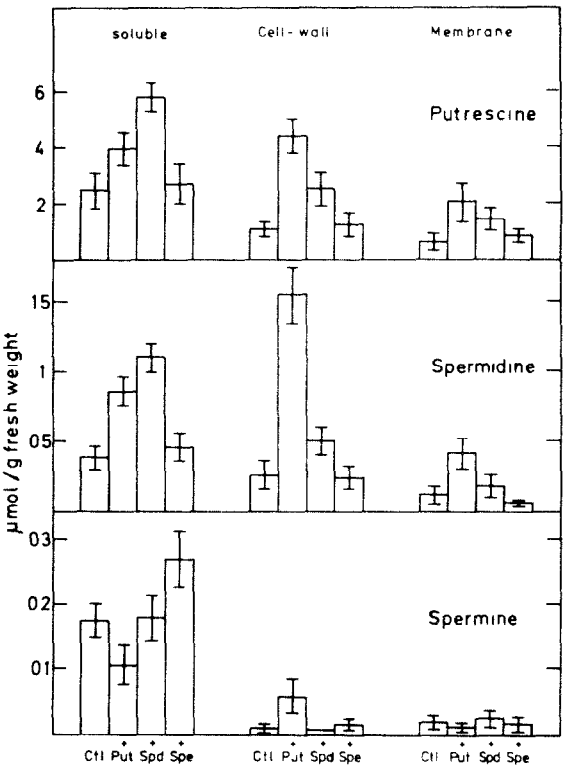

Fig 2 Polyamine titres in tobacco plants after exogenous supply of polyamines Polyamines were applied to the roots of tobacco Bel W 3 plants for two days Leaves were analysed for free and conjugated putrescine (upper panel), spermidine (centre) and spermine levels (lower panel) in the indicated cell fractions (soluble, cell wall or membrane-associated) Bars represent ± s e of 3–4 replicates

Putrescine supply to tobacco Bel W 3 plants led to a significant increase in cell wall-associated putrescine (4-fold) and spermidine (6-fold) levels. Thus, the levels of cell wall-associated putrescine approached those of soluble polyamines in control plants. Spermidine supply (+Spd; Fig 2) doubled putrescine and spermidine levels In control plants, polyamines in the 100 000 *g* membrane pellet were 5–15% of the total polyamine content. Putrescine supply increased the membrane-associated putrescine and spermidine levels two- to three-fold. Again, spermine titres were not influenced by either treatment.

Reaction rate constants

The reaction rate constants determined for the free polyamines with hydroxyl (OH), *tert*-butoxyl (*t*-BuO·), sulphite (SO_3^-) and superoxide/peroxyl ($O_2^-HO_2$) radicals are given in Table 1 To obtain rate constants with OH radicals, all polyamines were evaluated by pulse radiolysis using *p*-nitrosodimethylaniline as competitor [12] The closely similar values of the free polyamines are similar to other aliphatic amines [8]

The values for *t*-BuO radicals are included in Table 1 because the reactivity of this radical towards natural compounds has been shown to be representative for other alkoxyl radicals which may be formed during lipid peroxidation [10] The reactivity of *t*-BuO in aqueous solutions is rather high [10] and it attacks spermine, cadaverine and ornithine with rate constants of 4 2–6 0 $\times 10^6$ M^{-1} sec^{-1} while the other compounds are below the detection limit of the system (1.5×10^5 M^{-1} sec^{-1}) The relative values as determined by the 'crocin assay' in ref [13] were converted into absolute values based on the rate constant of *t*-BuO with crocin of 3×10^9 M^{-1} sec^{-1} [10]

Still slower is the reaction with SO_3^-, the predominant radical during sulphite autoxidation [14] The rate constants with spermine and cadaverine after pulse-radiolytic generation of SO_3^- [11], again using crocin as competitor, were smaller than 5×10^4 M^{-1} sec^{-1}, the detection limit for this system Putrescine gave a value of 2.6×10^5 M^{-1} sec^{-1}

The superoxide radical anion O_2^- as the most commonly encountered oxygen radical species in biological systems reacts with most substances quite slowly [15] The observed rate constants of $1.1–2.9 \times 10^2$ M^{-1} sec^{-1} at pH 7 5 with putrescine, spermidine and cadaverine reflect this poor reactivity Observing the decay of the pulse-radiolytically produced corresponding acid, HO_2, at pH 3 5, we find no reaction whatsoever with cadaverine and putrescine—the detection limit in this case being 10^4 M^{-1} sec^{-1} owing to the much faster dismutation reaction of HO_2 The small decrease of the O_2^- yield at high polyamine concentrations (data not shown), points to a limited reaction of hydrated electrons [e_{aq}^-, the nominal precursor of O_2^- according to eqn (4)] with the amines

Aside from the free form, the major part of polyamines are present in plant cells as conjugates with hydroxycinnamic acids [16–18] The respective rate constants of some radicals with three hydroxycinnamic acids and their mono-conjugates with putrescine are listed in Table 2.

Table 1. Rate constants of polyamines and related substances with different types of radicals

Substrate	Rate constant (M^{-1} sec^{-1}) OH* ($\times 10^{-8}$)	t-BuO·† ($\times 10^{-6}$)	$SO_3\cdot^-$‡ ($\times 10^{-4}$)	$O_2\cdot^-$ ($\times 10^{-3}$)	HO_2 ($\times 10^{-3}$)
Putrescine	1.13	<0 15§	25.7‖	0 11	<10§
Cadaverine	2 9	4.8	<5§	0.29	<10§
Spermidine	1 25	<0.15§	—	0.29	—
Spermine	1.30	4.2	<5§	—	—
Ornithine	0.75	6.0	—	—	—
Taurine	0 38	<0 15§	—	—	—

*Competition with p-nitrosodimethylaniline, $k_{(OH+p\text{-NDA})} = 1\,25 \times 10^{10}$ M^{-1} sec^{-1} (ref. [9]).
†Competition with crocin, $k_{(t\text{-BuO}\cdot + \text{crocin})} = 3 \times 10^9$ M^{-1} sec^{-1} (ref. [10]).
‡Competition with crocin, $k_{(SO_3^- + \text{crocin})} = 1 \times 10^9$ M^{-1} sec^{-1} (ref [11]).
§Not observable (value is detection limit of system)
‖Putrescine bleaches crocin at higher concentrations.
— Not determined

Table 2 Rate constants of putrescine, hydroxycinnamic acids and amide conjugates with different types of radicals

Substrate	Rate constant (M^{-1} sec^{-1}) OH ($\times 10^{-9}$)	t-BuO·* ($\times 10^{-8}$)	$SO_3\cdot^-$† ($\times 10^{-6}$)	O_2^- ($\times 10^{-4}$)
Putrescine‡	0.113	<0.0015	0.26	0.01
p-Coumaric acid	8.6§	0 75	—	—
Caffeic acid	24.1‖	1.02‖	4.5	40.0
Ferulic acid	6.1‖	0.90‖	—	—
p-Coumaroylputrescine	3.3§	1.68	—	—
Caffeoylputrescine	11.0§	1 02	7.0	8.5
Feruloylputrescine	13 0§	2.46	—	—

*Competition with crocin, $k_{(t\text{-BuO}\cdot + \text{crocin})} = 3 \times 10^9$ M^{-1} sec^{-1} (ref. [10]).
†Competition with crocin, $k_{(SO_3^- + \text{crocin})} = 1 \times 10^9$ M^{-1} sec^{-1} (ref. [11]).
‡Data from Table 1.
§Competition with t-butanol, $k_{(OH + t\text{-BuOH})} = 6 \times 10^8$ M^{-1} sec^{-1} (ref [9]).
‖Data from ref [19]
— Not determined.

The data clearly show that the radical scavenging effect of these conjugates resides in the phenolic hydroxy group and not in the amine moiety. In all instances, putrescine shows the lowest reactivity as compared to the hydroxycinnamic acids and conjugates. In the case of the hydroxycinnamic acids and their amide conjugates, transient absorption of radical intermediates could be directly observed (Fig. 3). These radicals are formed after ·OH attack and represent the different phenoxyl or semiquinone radicals. Intermediary formation of hydroxycyclohexadienyl radicals before water elimination takes place [20] was not observed. The fact that the conjugates show transient spectra similar to the hydroxycinnamic acids rather than the parent polyamines is again evidence that the site of attack is the phenolic hydroxy group for both types of substrates.

DISCUSSION

Polyamines as protectants against ozone damage

Treatment of the ozone-sensitive tobacco cultivar Bel W 3 with the diamine putrescine and the polyamines spermidine and spermine resulted in significant suppression of ozone-induced necrosis (Fig. 1). The results further support the previously observed 'antiozonant' effect [4]. In our experiments, polyamines were applied to the roots of tobacco plants to prevent direct effects on stomata. Alterations of stomatal activities occurred when isolated pea epidermis was treated with directly applied exogenous polyamines [21], with the possible consequence that the internal doses of gaseous pollutants in the plants are significantly changed [22].

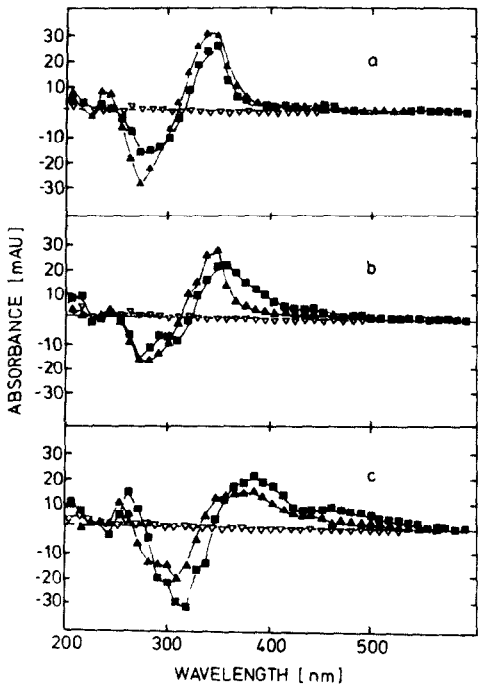

Fig 3 Transient spectra after attack of hydroxyl radicals Aqueous solutions saturated with N_2O, pH 6 5 (without buffer), irradiated at pulse doses of about 30 Gy. Uncorrected, dose-normalized transient spectra 35 μsec after the pulse (abscissa wavelength in nm, ordinate absorption values in milli absorption units, mAU). (■) Phenolic acids coumaric acid (49 μM), caffeic acid (55 μM), ferulic acid (50 μM), (▼) conjugates coumaroyl putrescine (120 μM), caffeoyl putrescine (65 μM), feruloyl-putrescine (34 μM), (△) putrescine (0 5 mM) (a) Coumaric acid and derivatives, (b) ferulic acid, (c) caffeic acid

Short distance and cellular transport of polyamines has been described [23]. Here, polyamine titres were measured in recently mature leaves which were most susceptible to ozone [24] following root application of polyamines. Both the contents of free putrescine and spermidine as well as their conjugates were increased after treatment with putrescine Spermidine application resulted not only in elevated spermidine content in the leaves, but also in the levels of putrescine. Further studies with radiolabelled polyamines are under way to establish the occurrence of long-range transport and to determine whether spermidine induces the formation of its precursor putrescine. Alternatively, putrescine could be formed by degradation of spermidine. The levels of free and conjugated polyamines attained by root application reached 1–10 μmol per g fresh weight of tobacco leaves. Polyamines in the cell wall and membrane-associated fraction were elevated up to two- to six-fold.

Polyamines in tobacco plants are predominantly conjugated with hydroxycinnamic acids [18]. In the tobacco cultivars Bel W 3 and Bel B, mono-substituted caffeoyl putrescine was the main amide conjugate. These conjugates are known to increase upon infection with tobacco mosaic virus or as a consequence of environmental stress factors [2, 17]. Total putrescine levels of tobacco Bel W 3 have been shown to be 50% lower than those found in the ozone-tolerant cultivar Bel B (Langebartels *et al*, in preparation). Ozone induced a rapid and prominent increase in the levels of both free and conjugated putrescine and the activity of arginine decarboxylase in Bel B plants. In the ozone-sensitive tobacco Bel W 3, this induction was very slow and reached its maximum only when symptom development had taken place These observations indicate that the protective effect of exogenously applied polyamines may also be exerted by induction of endogenous polyamine synthesis.

Polyamines as radical scavengers

When the rate constants of the free polyamines as given in Table 1 are compared with those of representative radical scavengers, which incidently are also good antioxidants, it is obvious that the reactivities of the polyamines are rather low and that they are poor candidates as radical scavengers (Table 3). These data extend the long-standing results on the low reactivities of aliphatic amines with ·OH radicals [8]

Radical scavenging properties of polyamines were proposed by Drolet *et al* [7], and a correlation was found with the extent of amination by these authors. This correlation was absent when OH radicals were generated selectively by pulse radiolysis (Table 1) It is thus likely that polyamines interfered with the generation of radicals in non-radiolytic systems, especially when assayed at high concentrations Ozone may react with uncharged species of amines [30] From the data in Table 1 and from the fact that polyamines are fully protonated at physiological pH, radical scavenging properties of free polyamines against oxyradicals derived from ozone can be excluded

In Table 2, rate constants with free radicals of putrescine conjugates with *p*-coumaric, caffeic and ferulic acid are compared with those of free putrescine and of the plant phenolic acids. Free acids and putrescine conjugates were similarly effective against specific radical species, whereas putrescine itself was far less active. Polyamine conjugates may therefore function as radical scavengers, but this property is exclusively due to the hydroxycinnamic acid moiety of the molecule A number of mono- and dihydroxylated phenolic acids have previously been shown to scavenge *t*-BuO and LO· radicals [13] Free ferulic acid and caffeic acid may be toxic to plant cells [31], yet their polyamine conjugates could contribute to scavenging effects as non-toxic derivatives

Taking the short lifetimes of OH or RO radicals into consideration, effective scavengers for these most reactive radicals should be localized near the site of radical generation. In plant tissues, radical scavenging compounds within the aqueous layer surrounding mesophyll cells, in the cell walls and associated with the plasmalemma are of special interest for a primary defence against ozone-derived oxyradicals Polyamines have been found to be associated with cell walls and membrane pellets (Fig. 2), but further studies are needed to show whether these compounds are bound as free polyamines (as is the case in mung bean hypocotyls where polyamines were shown to be linked to the uronide fraction of the cell wall, ref. [32]) or as amide conjugates with hydroxycinnamic acids

Generating the individual radicals by selective radiolytic and photolytic methods, we also find specific ranges of rate constants for each type of radical (Table 2) The

Table 3 Rate constants of representative radical scavengers with different types of oxygen radicals

Substance	Rate constant (M^{-1} sec^{-1}) ·OH $k(\times 10^{10})$	ref.	*t*-BuO·* $k(\times 10^{8})$	ref.	$O_2\cdot^-/HO_2$ $k(\times 10^{4})$	ref.
Ascorbate	1 1	[9]	22 0	[10, 13]	5.75	[25]
Diphenylamine	1.3	[9]	13 0	[10, 13]	—	—
Nordihydroguaiaretic acid	1.33	[19]	2.3	[10, 13]	—	—
Propyl gallate	1.2	[9]	2 0	[10, 13]	26.0	[26]
Quercetin	0.43	[27]	25.0†	[10]	8.9	‡
Trolox *c*§	3.45	[19]	6 9	[10, 13]	20.0: HO_2	[28]
					10^{-5}· O_2^-	[28]

Compilation of literature values for neutral aqueous solution, except where stated otherwise

*All values except for quercetin obtained as relative rate constants in the 'crocin assay' [13], absolute values based on $k_{crocin}+t$-BuO· of 3×10^9 M^{-1} sec^{-1} [10]; the rate constant for quercetin was obtained by direct evaluation of transient formation [10].

†Value at pH 11.5

‡Unpublished result from our laboratory

§2-Carboxyl-6-hydroxy-2,5,7,8-tetramethyl chromane = water-soluble model compound of vitamin E (ref [29]), data for HO_2 at pH 2, for O_2^- at pH 10 in ethanol–water (ref. [28]).

— No values known or determined.

differences (*ca* 20–30 fold for $SO_3\cdot^-$, 30–200 fold for ·OH, 500–1600 fold for *t*-BuO· and 850–4000 fold for $O_2\cdot^-$) do not reflect the electrophilicity of the radicals, as this would most likely be in the order of ·OH > *t*-BuO· ≫ $SO_3\cdot^-$ > $O_2\cdot^-$ [33]. It is generally assumed that the higher the electrophilicity, the faster and less discriminating a certain radical would react. Also, the fact that some rate constants of conjugates are higher than those of the hydroxycinnamic acids (or *vice versa*) is of minor importance, as all are in the range of diffusion-controlled reactions.

Ozone changes the permeability and integrity of plant membranes [5]. This deleterious effect is, at least in part, consistent with lipid peroxidation reactions. An antioxidative or radical-scavenging function of polyamines, as suggested by Drolet *et al.* [7] and, according to our results, confined to the hydroxycinnamic acid conjugates, implies that radical intermediates occurring during lipid peroxidation are scavenged.

An alternative process for blocking lipid peroxidation is to prevent the formation of radicals altogether by removing catalytic metal ions which otherwise could react with hydroperoxides and thus initiate lipid peroxidation via alkoxyl radicals [34]. This principle is invoked in the proposal of Tadolini [35]—based on earlier studies of Kitada *et al.* [36]—that polyamines inhibit lipid peroxidation by promoting iron chelation in a ternary complex with Fe^{3+} and the polar head groups of lipid membranes.

SYNOPSIS

From the above it is clear that polyamines can protect plants from ozone damage without being effective radical scavengers themselves. The effect may either derive from the radical scavenging properties of the hydroxycinnamic acid moieties of polyamine conjugates or by inhibition of lipid peroxidation. Alternatively, the protective effect would have to be attributed to indirect mechanisms, of which the following proposals are consistent with the existing literature. (i) The known endogenous protective system against ozone damage [6] may be activated by polyamines. (ii) Polyamines bind tightly to nucleic acids [2] and may thereby induce protective functions at a transcriptional level. They may also modulate enzyme activities post-transcriptionally by covalent linkage to glutamyl residues as has been shown for leaf protease [37]. Polyamines may thereby counteract processes of premature senescence caused by ozone. (iii) Polyamines bind to membrane lipids and may influence protective membrane functions which are generally known to be lipid-dependent [38]. Further research is clearly required to elucidate the mechanism of protection against ozone damage by polyamines.

EXPERIMENTAL

In vivo studies. Plants of the tobacco (*Nicotiana tabacum* L) cultivar Bel W 3 (O_3-sensitive) were grown in hydroponic culture in Perlite and Hoagland nutrient soln Plants were maintained in a controlled growth room under continuous light (10 klx, 35 W/m^2) at 25 ± 1° and 70 ± 5% relative humidity. Plants were used for experiments after 8 to 10 weeks of culture.

Putrescine, spermidine and spermine were root-administered to the nutrient solution, pH 5 6, for two days before ozone exposure (0.15 ppm, 5 hr) in plexiglass chambers O_3 was generated by electric discharge in dry O_2 (500 M, Fischer, Meckenheim) and was bled into filtered air Particle, activated charcoal, and permanganate (Purafil) filters were used to remove SO_2, O_3 and nitrogen oxides to concns below 0 005–0.015 ppm each [39]. Air in the chambers was sampled using Teflon lines, and concns of O_3 were measured with a CSI 3100 ozone analyser (Messer–Griesheim, Duisburg) calibrated with Dasibi Model 1009-CP analyser. Plants were returned to the growth chamber after the treatment and after 48 hr the necrotic area of leaves three and four from the top (leaf 1 longer than 8 cm) was determined with a planimeter (Delta T).

Samples of leaves 3 and 4 (0 2 g) were homogenized in liquid nitrogen and were extracted in 5 ml 5% (v/v) $HClO_4$ Free

polyamines were determined in the supernatant after centrifugation (20 000 *g* for 20 min) Soluble conjugated polyamines were analysed after hydrolysis of this supernatant in 6 M HCl (16 hr, 110°) For the analysis of cell wall-bound and membrane-associated polyamines, cells were homogenized in 50 mM HEPES–NaOH buffer, pH 7 5, and centrifuged at 6000 *g* for 10 min to sediment cell wall material. The pellet was washed twice with the same buffer Cell walls were purified according to ref [40] and were then hydrolysed as above The supernatant was centrifuged at 100 000 *g* for 60 min and the membrane pellet hydrolysed as above after two washings with HEPES buffer Polyamines were dansylated according to ref [41] Dansylated products were extracted with toluene and separated by HPLC on a Spherisorb ODS II 5 μm column (250 × 4 6 mm) with a MeOH–H_2O gradient (65–95% MeOH, 20 min) at a flow rate of 1 ml/min Polyamine contents were determined using a spectrofluorimeter at 360 nm (excitation) and 510 nm (emission)

Irradiation studies These experiments were performed using a Febetron 705 accelerator (Hewlett–Packard) generating 2 MeV electrons and a pulse radiolysis set-up described earlier [42] Reaction rate constants of various polyamines, hydroxycinnamic acids and mutual conjugates in neutral aqueous solution prepared with 'Milli-Q' water were determined with hydroxyl, *tert*-butoxyl, superoxide and sulphite radicals as representative species of biologically and/or environmentally important radicals Pulse-radiolytic methods for selective generation of radicals were used to convert the primary water radicals, OH, e^-_{aq} (hydrated electrons), H (hydrogen atoms), yielding in turn [43] (i) 90% OH and 10% H·, if the solns were saturated with N_2O to convert e^-_{aq} into additional OH radicals

$$e^-_{aq} + N_2O + H_2O \rightarrow OH + OH^- + N_2 \quad (1)$$

At a commonly used pulse dose of *ca* 30 Gray and a maximal radiolytic yield for the sum of the water radicals of 6 4 molecules per 100 eV of absorbed energy [43], the total radical concn at the end of the 40 nsec pulse amounted to 20 μM

(ii) 100% $O_2^{\cdot -}$ radicals, if solns containing sodium formate (10 mM) were saturated with O_2

$$HCOO^- + OH \rightarrow COO^{\cdot -} + H_2O \quad (2)$$

$$COO^{\cdot -} + O_2 \rightarrow O_2^{\cdot -} + CO_2 \quad (3)$$

$$e^-_{aq} + O_2 \rightarrow O_2^{\cdot -} \quad (4)$$

$$H + O_2 \rightarrow HO_2 \rightleftharpoons O_2^{\cdot -} + H^+ \quad (5)$$

$O_2\cdot^-$ radicals were generated in 2 mM phosphate buffer, pH 7 5, containing 0 1 mM EDTA and HO_2 in solns acidified with $HClO_4$ to pH 3 5

(iii) 90% $SO_3^{\cdot -}$ and 10% H radicals, if solns containing $NaHSO_3$ (50 mM) were saturated with N_2O, equation (1), followed by

$$HSO_3^- + OH \rightarrow SO_3^{\cdot -} + H_2O \quad (6)$$

Ten-fold lower radical concns were used in this system as the competitor crocin, owing to the extremely high molar absorptivity [10], could be employed only up to a concn of 3–4 μM

Photochemical generation of *t*-BuO was achieved by homolytic cleavage of *t*-BuOOH with 254 nm UV light in the presence of *t*-BuOH to scavenge the simultaneously produced OH radicals

$$t\text{-BuOOH} \rightarrow t\text{-BuO} + OH \quad (7)$$

Because of the low rate constants, competition experiments were evaluated [12], using the competitors listed in the footnotes to Tables 1 and 2 In the case of $O_2^{\cdot -}/HO_2^{\cdot}$, the slow decay of the absorption at 260 nm was evaluated

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