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PHOTODEGRADATION OF CHLOROPHYLL PHYTYL CHAIN IN SENESCENT LEAVES OF HIGHER PLANTS

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Key Word Index—*Avena sativa*; Gramineae; *Petroselinum sativum*; Umbelliferae; senescence; singlet oxygen; phytyl chain photodegradation.

Abstract—6,10,14-Trimethylpentadecan-2-one, 3-methylidene-7,11,15-trimethylhexadecan-1,2-diol, (*Z* and *E*) 3,7,11,15-tetramethyl-2,3-epoxyhexadecanols, (*Z* and *E*) 3,7,11,15-tetramethyl-2,3-epoxyhexadecanals and (*Z* and *E*) 3,7,11,15-tetramethylhexadec-2-enals were unambiguously identified in attached leaves of senescing *Avena sativa* collected during late summer. These isoprenoid compounds result from the photooxidation of the chlorophyll phytyl chain during senescence. *In vitro* dark and light-induced senescence experiments carried out with *Petroselinum sativum* confirmed these results.

INTRODUCTION

Despite the importance of chlorophyll in photosynthesis, surprisingly little is known about its breakdown during leaf senescence [1]. Photochemical processes may play a major role in this pigment loss [2]. Indeed, if *in vivo* the chloroplast environment (ascorbate, glutathione, α-tocopherol, β-carotene) protects chlorophyll from toxic oxygen species $O_2^{\bullet -}$, $HO^\bullet$, $^1O_2$, $ROO^\bullet$) produced during photosynthesis [3], in senescing cells the rate of formation of these oxygen species exceeds the quenching capacity of chloroplasts and the photodegradation of chlorophyll can occur [4] (Fig. 1).

Data about photochemical degradation of chlorophyll are very scarce in the literature [5–7] and concern essentially the tetrapyrrolic moiety of the molecule. Recently, we demonstrated in dead phytoplankton cells that photochemical processes act also intensively on the phytyl side chain of chlorophyll-*a* and chlorophyll-*b* [8], which has been often neglected in previous studies of the breakdown of chlorophyll [9]. These reactions involving mainly singlet oxygen [10] and to a small degree peroxy radicals [11] operate on free and esterified phytol (Fig. 1).

The aim of the present work was to extend these results to terrestrial higher plants, where chlorophyll turnover is considerably slower than in phytoplankton.

RESULTS AND DISCUSSION

1. The Avena sativa *system*

GC/MS analyses of fractions F_B and F_C obtained after extraction of senescing attached leaves of *Avena sativa* revealed the presence of significant quantities of phytol **1**, 6,10,14-trimethyl-pentadecan-2-one **2** and 3-methylidene-7,11,15-trimethylhexadecan-1,2,-diol **3**, accompanied by small amounts of (*Z* and *E*) 3,7,11,15-tetramethyl-2,3-epoxyhexadecanols **4** and **5**, (*Z* and *E*) 3,7,11,15-tetramethyl-2,3-epoxyhexadecanals **6** and **7** and (*Z* and *E*) 3,7,11,15-tetramethylhexadec-2-enals **8** and **9** (Table 1). Saponification of these senescing attached leaves yields only compounds **1**–**5** in higher quantities (Table 1); epoxy-aldehydes **6** and **7** and α,β-unsaturated aldehydes **8** and **9** do not survive alkaline hydrolysis and are degraded mainly to ketone **2** (Fig. 2) [10, 12].

While compounds **2**–**5** were previously identified in senescing planktonic cells [8], to our knowledge this is the first isolation of isoprenoids **6**–**9** from biological material. The absence of compounds **3**–**9** in young *A. sativa* leaves collected in June and allowed to senesce in darkness for three months clearly establishes their photochemical origin. On the other hand, we detected 20 $\mu g\, g^{-1}$ fr. wt of ketone **2** in the fraction F_B and among unsaponifiable lipids of this dark manipulation. This free isoprenoid ketone is also present in similar concentration in non-senescing attached leaves of *A. sativa*. This surprising result can be attributed to a bacterial degradation of free phytol released by chlorophyllase in the non-senescing plant. Indeed, some

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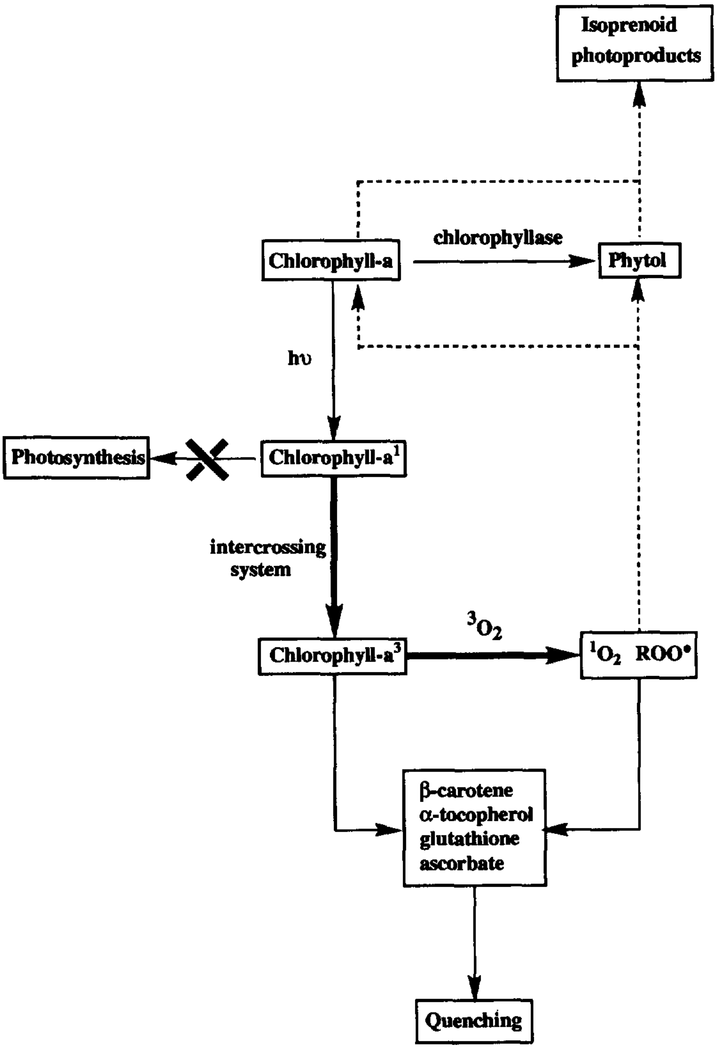

Fig. 1. Proposed scheme for the photodegradation of the phytyl chain of chlorophyll during plant senescence.

authors have previously observed the production of such ketone after incubation of phytol with bacteria [13]. Taking into account the higher quantity of ketone **2** detected among the unsaponifiable lipids of senescing attached leaves of *A. sativa* (547 μg g^{-1} fr. wt) (Table 1), it can be concluded that photochemical processes constitute the major source of this isoprenoid ketone (Table 1).

The formation of the two isomeric epoxy-alcohols **4** and **5** involves the addition of peroxy radicals (ROO•)

Table 1. Isoprenoid compounds identified in attached senescing leaves of *Avena sativa* collected in September

Compound	Code	Concentration (μg g^{-1} fr. wt) in free form	Concentration (μg g^{-1} fr. wt) after saponification
Phytol	**1**	57	1890
6,10,14-Trimethylpentadecan-2-one	**2**	34	547
3-Methylidene-7,11,15-trimethylhexadecan-1,2-diol	**3**	24	431
(*Z*) 3,7,11,15-Tetramethyl-2,3-epoxyhexadecanol	**4**	1	6
(*E*) 3,7,11,15-Tetramethyl-2,3-epoxyhexadecanol	**5**	3	48
(*Z*) 3,7,11,15-Tetramethyl-2,3-epoxyhexadecanal	**6**	3	—
(*E*) 3,7,11,15-Tetramethyl-2,3-epoxyhexadecanal	**7**	4	—
(*Z*) 3,7,11,15-Tetramethylhexadec-2-enal	**8**	3	—
(*E*) 3,7,11,15-Tetramethylhexadec-2-enal	**9**	7	—

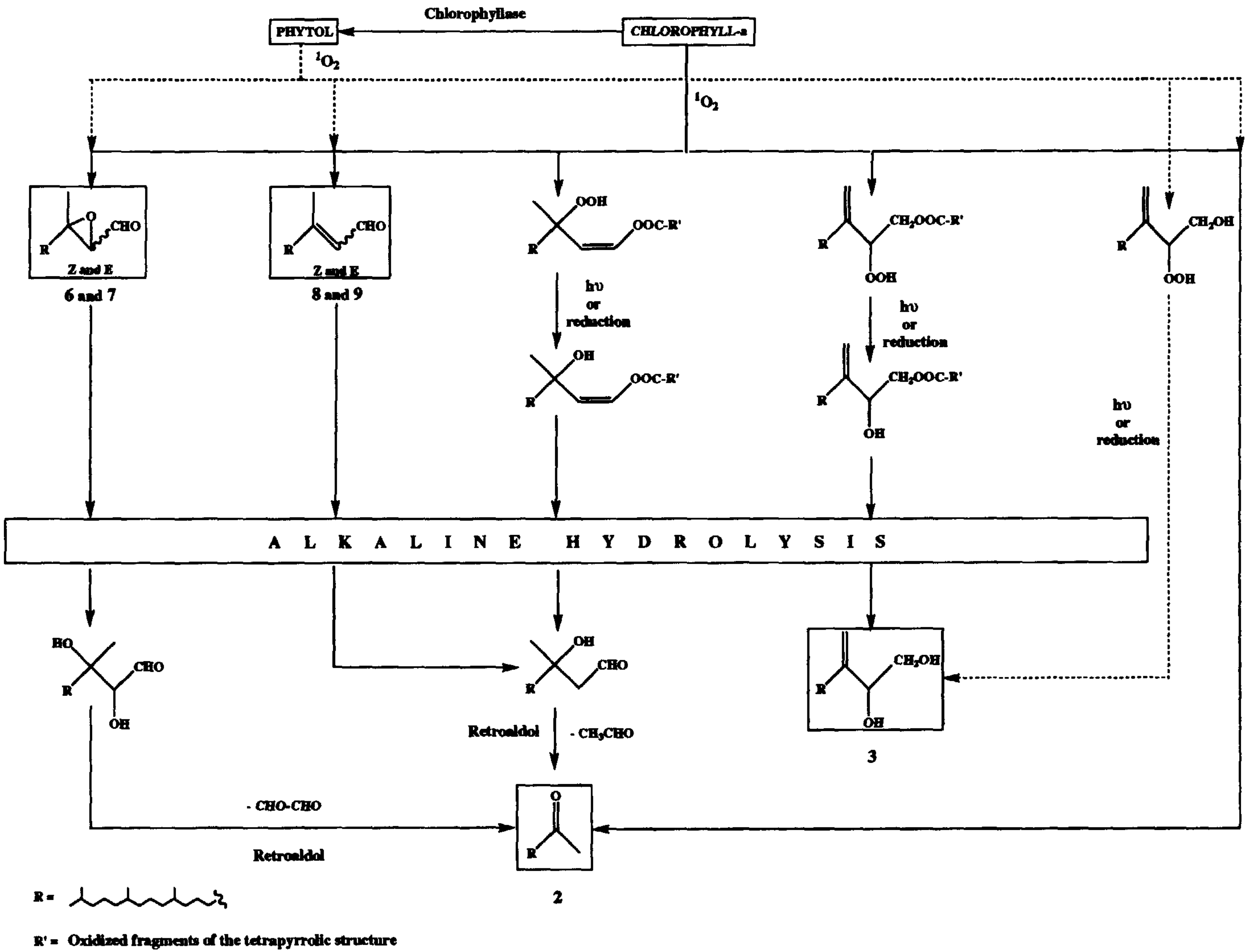

Fig. 2. Synthetic scheme showing the main photoproducts resulting from an attack of $^1O_2$ to the olefinic carbons of the phytyl chain of chlorophyll and their behaviour during alkaline hydrolysis.

to the double bond of phytol or chlorophyll phytyl chain [11]. The other compounds **2**, **3**, **6**, **7**, **8** and **9** originate from an attack of singlet oxygen ($^1O_2$) on the olefinic carbons of phytol or chlorophyll phytyl chain. These processes, which were previously described [10, 14] are summarized in Fig. 2.

The quantitative results obtained (Table 1) show that during light-induced senescence of *A. sativa* (where an important chlorophyllase activity was demonstrated [15]) the major part of chlorophyll is photodegraded before hydrolysis of the phytyl chain.

2. *The* Petroselinum sativum *system*

In order to compare the photochemical fate of phytyl chain of chlorophyll in species which differ markedly in their capacity of chlorophyll hydrolysis during foliar senescence [15–17], we used *Petroselinum sativum*. Detached leaves of fresh parsley were allowed to senesce in darkness or in light for different periods. During light-induced incubation parsley leaves underwent yellowing and then browning without notable fungal infection, while under darkness leaves remained green. After irradiation, we detected very small quantities of free isoprenoid compounds; this is in good agreement with the well known low chlorophyllase activity of parsley [16, 17]. On the other hand, saponification gives high quantities of ketone **2** and diol **3** (Table 2), which are not produced during senescence in darkness.

It seems that in the case of *P. sativum* the photodegradation of the chlorophyll phytyl chain involves solely singlet oxygen (we failed to detect traces of epoxy-alcohols **4** and **5**, which are characteristic of an attack by peroxy radicals [11]). Losses of phytyl chain increase with photodegradation (Table 2). This can be attributed either to the involvement of secondary photochemical processes which generate volatile photoproducts or to the increased polarity of the photooxidized tetrapyrrolic structure (R′ in Fig. 2) which can decrease the efficiency of the extraction.

As in the case of phytoplankton [8] photochemical processes act significantly on the chlorophyll phytyl chain during light induced senescence of terrestrial higher plants. In attached leaves of senescing *A. sativa* these processes involved mainly singlet oxygen and to a minor degree peroxy radicals, while in detached leaves of *P. sativum* only singlet oxygen seems to intervene. Even in *A. sativa* (where a relatively high chlorophyllase activity was demonstrated [15]) the major part of the chlorophyll phytyl chain is photodegraded before hydrolysis of the ester bond. Taking into account the great quantity of chlorophyll-*a* present on land [18], we conclude that important amounts of the isoprenoid photoproducts described in this work must be produced annually during natural senescence of terrestrial higher plants.

EXPERIMENTAL

Plant material. Leaves of *A. sativa* were collected from young (uniformly green) and senescing plants growing on campus during springtime (June) and late summer (September). Parsley (*P. sativum*) leaves were purchased fresh in the market.

Induction of senescence. 1 g of leaf segments were allowed to senesce at 25° either in light or in darkness in non-sterilized petri dishes containing dry filter paper. Irradiations were carried out with a 30 W fluorescent lamp (Osram, daylight).

Extraction and reduction. Leaf segments were placed in a mortar and ground in the presence of MeOH (20 ml per g). The extraction was repeated twice and the hydroperoxides were reduced by shaking the combined extracts and the debris with $(CH_3)_2S$ (1 ml) overnight at 0°.

Isolation of free isoprenoid compounds. After reduction, the mixt. was filtered, evapd to dryness under vacuum and dried azeotropically with Me_2CO. The residue obtained was chromatographed on a wet packed (*n*-hexane) column filled with silica gel (Kieselgel S + 3% H_2O). Three frs were eluted with 100 ml of *n*-hexane, 100 ml of $CHCl_3$ and 50 ml of MeOH, respectively: F_A containing hydrocarbons; F_B containing alcohols, ketones, aldehydes, epoxy-aldehydes and epoxy-alcohols; and F_C containing diols and acidic compounds. All manipulations were carried out with foil-covered vessels in order to exclude photochemical artifacts.

Isolation of total (free + esterified) isoprenoid compounds. After reduction, 40 ml of H_2O and 5.6 g of KOH were added to the entire mixt. (debris + MeOH extracts + $(CH_3)_2S$) and the whole was heated under reflux for 2 hr. The soln was extracted 3 times with *n*-hexane to give unsaponifiable lipids. The combined extract were dried on Na_2SO_4, filtered and evapd to dryness.

Derivatizations. Unsaponifiable extract and frs F_B and F_C were acetylated in 300 μl of a mixt. of pyridine and Ac_2O (2:1) for 2 hr. After destruction of the

Table 2. Changes in phytyl chain of chlorophyll during *Petroselinum sativum* incubation in light or in darkness

Time (days)	Light exposure (Ein m^{-2})	Concentration of **1** (μmol g^{-1} fr. wt)	Concentration of **2** (μmol g^{-1} fr. wt)	Concentration of **3** (μmol g^{-1} fr. wt)
0	0	3.5	0	0
15	65	2.8	0.1	0.1
30	130	1.4	0.5	0.5
54	233	0.8	0.6	0.7
54	0	3.3	0	0

excess of anhydride with MeOH (500 μl) and evapn to dryness under N_2, the residue was taken in hexane and analysed by GC MS. In the case of frs F_C subsequent silylation with bis-(trimethylsilyl)-trifluoroacetamide was necessary owing to the presence of acidic compounds.

Identification and quantitation of isoprenoid compounds. Photoproducts of phytyl chain were identified by comparison of their retention times and mass spectra with those of synthesized standards. Quantitative determinations were based on integrator data, which were calibrated with external standards. GC EIMS analyses were performed with a HP 5890 series II Plus gas chromatograph connected to a HP 5972 mass spectrometer. The following operating conditions were used: 30 m × 0.25 mm (i.d.) capillary column coated with DB5; oven temp. programmed from 60° to 130° at 30° min^{-1} and then from 130° to 300° at 3° min^{-1}; carrier gas pressure (He), 1.04 bar; injector temp, 280°; electron energy, 70 eV; source temp, 170°. Positive chemical ionization mass spectra were recorded on a HP 5987 mass spectrometer. Operating conditions: electron energy, 150 eV; CH_4 pressure, 0.2 Torr; source temp, 180°.

Standard compounds. Phytol **1** was purchased from Riedel de Haën. EIMS of acetate *m/z* (rel. int.): 278 $[M - CH_3COOH]^+$ (5), 123 (53), 95 (73), 82 (80), 81 (67), 68 (89), 57 (65), 55 (55), 43 (100), 41 (62). CI (CH_4)MS of acetate *m/z* (rel. int.): 339 $[M+H]^+$ (7), 279 $[M - CH_3COOH + H]^+$ (40). 6,10,14-*Trimethylpentadecan*-2-*one* **2** was synthesized by oxidation of phytol with $KMnO_4$ in Me_2CO [19]. EIMS *m/z* (rel. int.): 268 $[M]^+$ (0.5), 250 $[M - H_2O]^+$ (3), 124 (8), 109 (17), 95 (15), 85 (22), 71 (41), 58 (77), 43 (100). CI (CH_4)MS *m/z* (rel. int.): 309 $[M + C_3H_5]^+$ (7), 297 $[M + C_2H_5]^+$ (31), 269 $[M + H]^-$ (100). 3-*Methylidene*-7,11,15-*trimethylhexadecan*-1,2-*diol* **3** was produced in 2 steps from phytol according to a previously described procedure [14]. EIMS of di-acetate *m/z* (rel. int.): 294 (3), 276 $[M - 2CH_3COOH]^+$ (2), 126 (14), 121 (13), 111 (9), 84 (27), 71 (14), 57 (18), 55 (15), 43 (100). CI (CH_4)MS of di-acetate *m/z* (rel. int.): 397 $[M + H]^-$ (3), 337 $[M - CH_3COOH + H]^+$ (100). Epoxidation of a mixture of the two isomers of phytol (Fluka) with *meta*-chloroperoxy-benzoic acid in dry CH_2Cl_2 yielded (Z *and* E) 3,7,11,15-*tetramethyl*-2,3-*epoxyhexadecanols* **4** and **5** [20]. Mass spectra of these two isomers acetylated are similar. EIMS *m/z* (rel. int.): 312 $[M - CH_2{=}C{=}O]^+$ (2), 125 (6), 111 (10), 97 (12), 87 (100), 69 (13). CI (CH_4)MS *m/z* (rel. int.): 395 $[M + C_3H_5]^+$ (5), 383 $[M + C_2H_5]^+$ (9), 355 $[M + H]^+$ (100). (Z *and* E) 3,7,11,15-*tetramethyl*-2,3-*epoxyhexadecanals* **6** and **7** were synthesized by oxidation of the corresponding α,β-epoxy alcohols **4** and **5** with CrO_3-pyridine in dry CH_2Cl_2 [20]. Mass spectra of *Z* and *E* isomers are similar. EIMS *m/z* (rel. int.): 295 $[M - CH_3]^+$ (20), 281 (5), 207 (6), 111 (11), 97 (13), 85 (100), 71 (31), 69 (22), 57 (47). CI (CH_4)MS *m/z* (rel. int.): 351 $[M + C_3H_5]^+$ (5), 339 $[M + C_2H_5]^+$ (21), 311 $[M + H]^+$ (100). Oxidation of the two isomers of phytol with CrO_3-pyridine in dry CH_2Cl_2 gave (Z *and* E) 3,7,11,15-*tetramethylhexadec*-2-*enals* **8** and **9** [21]. EIMS of **8** *m/z* (rel. int.): 179 (4), 149 (27), 139 (17), 123 (15), 111 (37), 97 (63), 95 (35), 84 (100), 69 (26), 57 (28), 55 (42). CI (CH_4)MS of **8** *m/z* (rel. int.): 335 $[M + C_3H_5]^+$ (10), 323 $[M + C_2H_5]^+$ (35), 295 $[M + H]^+$ (100). EIMS of **9** *m/z* (rel. int.): 179 (3), 149 (16), 140 (15), 123 (12), 121 (13), 111 (33), 97 (40), 84 (100), 71 (15), 69 (16), 57 (21), 55 (30). CI (CH_4)MS of **9** *m/z* (rel. int.): 335 $[M + C_3H_5]^+$ (13), 323 $[M + C_2H_5]^-$ (37), 295 $[M + H]^+$ (100).

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