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Changes in nonpolar aldehydes in bean cotyledons during ageing

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Abstract

Ageing of plant organs is accompanied by an increased production of free radicals what results in membrane lipid peroxidation. Non-polar aldehydes originating from this process interact with the cellular material to form the fluorescent end-products, lipofuscin-like pigments (LFP). Their formation was studied both qualitatively and quantitatively in ageing of bean cotyledons. The concentration of lipofuscin-like pigments increased 9-fold in 14-d-old (senescent) cotyledons in relation to 8-d-old (young) cotyledons. HPLC fractionation patterns indicate changes in their composition during ageing. The LFP increase in old cotyledons was accompanied by elevated levels of non-polar aldehydes that increased during ageing to 167 %. The composition of aldehydes was studied by mass spectrometry. The most abundant fraction in both young and old cotyledon was represented by C12 aldehydes, which comprised both saturated and unsaturated species. We have observed differences in abundances of individual aldehydes between the young and the old cotyledons that might explain the differences in the composition of lipofuscin-like pigments. These results support the involvement of free radicals in plant ageing; however, it is suggested that plant aldehydic products of lipid peroxidation differ from those found in animals.

Additional keywords: lipid peroxidation, lipofuscin-like pigments (LFP), mass spectrometry, *Phaseolus vulgaris*.

Introduction

Ageing in plants is accompanied by the accumulation of the fluorescent end-products of lipid peroxidation, the so called lipofuscin-like pigments (LFP). The precise composition of these compounds is not known to date, because they form complex multicomponent mixtures that are difficult to separate and analyse. Previous studies have deduced that LFP might originate from the reactions of aldehydic products of lipid peroxidation with compounds containing primary amino-group (Chio and Tappel 1969, Esterbauer *et al.* 1986, Yin 1996, Choudhury *et al.* 2004, Gerstbrein *et al.* 2005).

Early studies confirmed increased lipid peroxidation in senescent leaves and a correlation between lipid peroxidation and increased membrane permeability (Dhindsa *et al.* 1981). LFP were observed both in animals (Sohal and Allen 1986) and in plants. The first observation of LFP in plant tissue was made in ripening pear and banana fruits (Maguire and Haard 1975). We found increased levels of LFP in chloroplasts prepared from the leaves of ageing beans (Wilhelm and Wilhelmová 1981). Accumulation of liposoluble fluorescent products was observed also in microsomal membranes from senescing bean cotyledons (Pauls and Thompson 1984) and in the leaves of a light-sensitive chlorescence pea mutant (Merzlyak *et al.* 1984). The treatment of the leaves of rice, amaranth, and maize with a free radical inhibitor, α-tocopherol, inhibited the formation of LFP (Wang *et al.* 1988). Fluorescent lipids have also been found in cytosol which contains peroxidized free fatty acids (Yao *et al.* 1993). In senescent cotyledons, the percentage of LFP decreased in cytoplasm and increased in microsomal membranes (Hudak *et al.* 1995). We have characterized LFP in ageing bean cotyledons and their time-course after decapitation of the plant epicotyl (Wilhelmová *et al.* 1997, 2004).

A relatively specific group of aldehydes is the non-polar species formed after free radical oxidation of unsaturated fatty acids in the process of membrane lipid peroxidation. These compounds can be easily

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Abbreviations: DNPH - 2,4-dinitrophenylhydrazine; LFP - lipofuscin-like pigments; TLC - thin layer chromatography.
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distinguished by their solubility from the water-soluble polar aldehydes which represent intermediates in plant metabolism not related to free radical reactions. Therefore we aimed the present study at the aldehydic compounds found in the chloroform-methanol extract from young and old bean cotyledons. We measured the total concentration of aldehydes, resolved them into several fractions by TLC, and analysed specific fractions using mass spectrometry. The results indicated an increase in non-polar aldehydes during cotyledon ageing which is accompanied by specific changes in aldehyde composition. These changes were concomitant with an increase in LFP, indicating a functional relationship.

Materials and methods

Plants: Bean (*Phaseolus vulga*ris L. cv. Jantar) cotyledons were used for our experiments. Plants were grown in plastic pots filled with sterilised sand in a growth chamber (*Klimabox 1300*, *Kovodružstvo*, Slaný, Czech Republic) at a day/night temperature of 18/16 °C, air humidity 60/80 % and irradiance of 220 μmol m^{-2} s^{-1} (PAR) during the 16-h photoperiod. Plants were watered daily by distilled water and twice a week by the Hewitt nutrient solution (for detail see Procházková and Wilhelmová 2004). The cotyledons were harvested at a plant age of 8 d (young) or 14 d (old).

Preparation of chloroform extracts: About 1 g of frozen cotyledons was cut into small pieces and extracted in 6 cm^3 of chloroform-methanol mixture (2:1, v/v) on a motor-driven shaker for 1 h. Then 2 cm^3 of water was added to achieve a phase separation, the sample was centrifuged and the lower chloroform layer was collected. The chloroform fraction was then washed twice with 2 cm^3 of water and was used for further measurements.

Assay of LFP: The washed chloroform extract was used for the measurement of the tridimensional fluorescence spectra. The excitation spectra were measured in the range of 300 - 400 nm for emission adjusted between 380 - 460 nm with a step of 5 nm. The quantitative estimation of LFP was based on the excitation and emission maxima found in 3D spectral arrays. The fluorometer was calibrated with the standard No. 5 of the instrument manufacturer. For the determination of LFP concentration wavelengths 355/400 nm (excitation/ emission) were used and expressed as relative fluorescence units (rfu) g^{-1}(f.m.).

HPLC analysis: LFP were resolved in a mixture of acetonitrile-methanol-water (5:2:3, v/v) on a *Shimadzu LC-9* (Kyoto, Japan) HPLC instrument equipped with a fluorescence detector set at 355/400 nm (excitation/ emission). A *C18* column (3 × 150 mm, *Tessek*, Prague, Czech Republic) was used with an elution rate of 0.5 cm^3 min^{-1}. For the separation hydrazones the same mobile phase was used. Another *C18* column (8 × 250 mm) gave optimum separation at 1.3 cm^3 min^{-1}. The fluorescence detector was adjusted at 350/410 nm.

Derivatization of aldehydes: 0.2 cm^3 of dinitrophenyl-hydrazine reagent (5 mg DNPH per 1 cm^3 in 1 M HCl) was added to 0.2 cm^3 of chloroform extract and allowed to react for 2 h at room temperature in the dark. Absorbance was measured at 360 nm, blank of chloroform-methanol mixture (2:1, v/v) and a coefficient of absorbance of 25 500 M^{-1} cm^{-1} was used (Beckman *et al.* 1991).

TLC analysis of hydrazones: 0.05 cm^3 of the samples of hydrazones was applied to silica-gel plates (*Alugram Sil G*, 10 × 10 cm, *Macherey-Nagel*, Düren, Germany). The plate was developed in 100 % dichloromethane in one direction, for the second direction ethyl acetate - dichlormethane mixture (1:10, v/v) as a developing solvent was used.

Sample preparation and the mass-spectrometry analysis: The individual spots were scratched off from the TLC plate, the sample was dissolved in 0.075 cm^3 of methanol, separated from the silica by centrifugation and used either for HPLC analysis or for mass-spectral analysis. The sample for mass-spectral analysis was evaporated under the stream of nitrogen.

Positive electron impact (EI) mass spectra and tandem mass spectra were obtained with a *VG AutoSpec* (*Micromass*, Manchester, UK). The ion source temperature was maintained at 220 ºC and the ionising voltage was 70 eV. Tandem mass spectra were obtained by selecting the desired ion with the EB section of the mass spectrometer and colliding it at 8 kV with sufficient Ar in the collision cell as to reduce the selected ion intensity by approximately 50 %. The resulting product ions were determined by a scan of the second electric sector. Data acquisition was carried out with a *VG OPUS 2.1C* data system interfaced to the mass spectrometer through a *VG SIOS* unit.

Statistical analysis: The data reported are the means of three measurements. The analysis of variance was calculated using the statistical analysis program *ANOVA* (*StatView*, *Abacus Concepts*, Berkeley, USA). The statistical significance of the observed differences was evaluated by the post-hoc test of Scheffé´s procedure.

Results and discussion

LFP in young and old cotyledons: The aldehydic products of lipid peroxidation form fluorescent end products, LFP. As the fluorescence measurement is generally very sensitive, it can serve for the detection of the production of aldehydes. The tridimensional spectral arrays represent a "fingerprint" for a given compound and can reveal subtle changes in LFP composition between the young (8 d) and old (14 d) cotyledons (Fig. 1) Emission maxima were determined from two-dimensional presentation (Fig. 1 *insets*). The young plants had fluorophores emitting at 400, 416, and 448 nm. In the old plants a fluorophore with an emission maximum at 410 nm became the predominant species.

Moreover, the samples differed in LFP concentration: 8-d-old samples contained 0.020 ± 0.005 r.f.u. g^{-1}(f.m.), 14-d-old cotyledons 0.180 ± 0.025 r.f.u. g^{-1}(f.m.). This represents an increase to 900 % ($P < 0.001$).

The fluorophores were resolved by HPLC into several

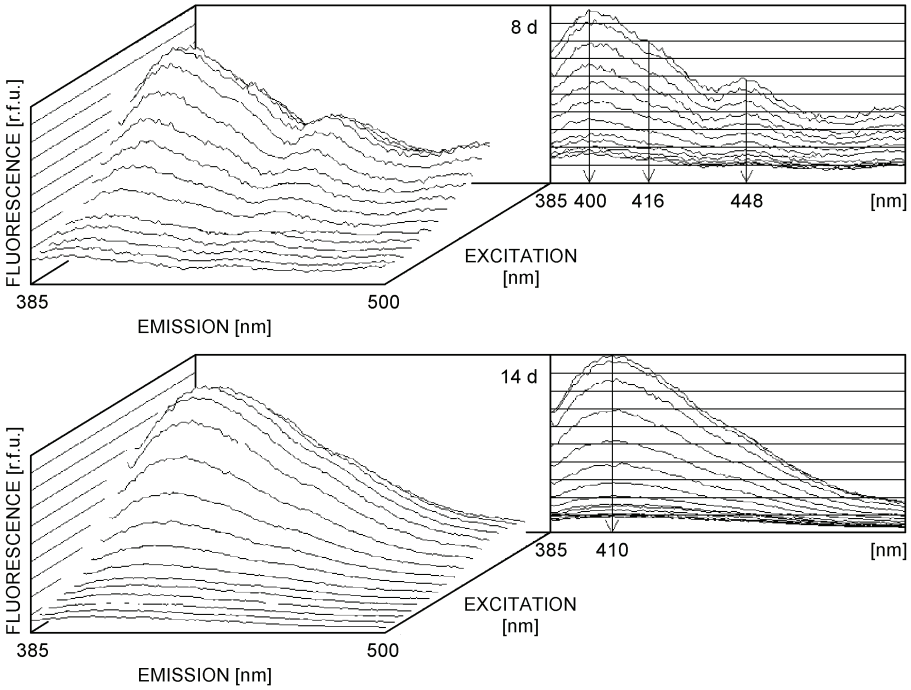

Fig. 1. Tridimensional spectral arrays of fluorescence emission spectra of the chloroform extracts from young (8 d) and old (14 d) cotyledons. The arrows in the 2D presentation (inserts) indicate emission maxima. The sensitivity of the fluorometer was adjusted for the maximum resolution of emission peaks and therefore different panels cannot be quantitatively compared.

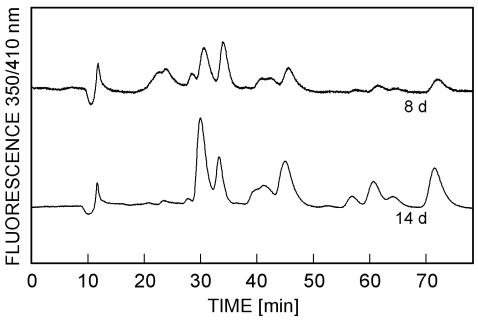

Fig. 2. HPLC analysis of LFP fluorophores. Upper trace 8 d old cotyledons, lower trace 14 d old cotyledons. The detector was set at 350/410 nm (excitation/emission).

fractions (Fig. 2). The fractionation pattern differed in the relative abundancies of individual peaks, indicating changes in LFP composition during ageing. Besides that, new fractions were observed in 14-d-old cotyledons. The early model experiments indicated the involvement of aldehydes originating from lipid peroxidation in LFP formation (Kikugawa *et al.* 1981). Among them, the long chain aldehydes, such as 4-hydroxynonenal, especially, were shown to interact with proteins to produce fluorescent compounds with characteristics of LFP (Bolgar and Gaskell 1996). Thus, an increased amount of free long-chain aldehydes creates a pre-requisite for LFP formation.

Quantification of non-polar aldehydes: As the fluorescence measurements provided indirect evidence for increased production of aldehydes during ageing, we attempted more direct proof of their presence. For the detection of aldehydes, the chloroform extract was

reacted with 2,4-dinitrophenylhydrazine. The quantitative expression gave 43.5 ± 0.7 μmol(aldehydes) g^{-1}(f.m.) in the young cotyledons, and 72.1 ± 1.2 μmol g^{-1}(f.m.) in the old cotyledons. This is an increase to 167 % ($P < 0.001$).

The hydrazones prepared from young and old cotyledons were resolved by TLC. The fractionation pattern did not differ between young and old cotyledons. The fraction 1, which represented the most intensive spot, was used for further characterisation by mass spectrometry. It was labeled 8-1 in young cotyledons, and 14-1 in old cotyledons.

Mass-spectrometric characterisation of aldehyde fractions: EI mass spectra revealed differences between fractions 8-1 and 14-1 (Fig. 3). The peaks labeled with *arrows* correspond to the hydrazones that could be identified in the mass spectra accordingly with the general formula of the aldehydes $C_nH_{2n}O$. In this way, for example, the peak at m/z 336 would correspond to the hydrazone of decanal. Another possibility that should be considered is the presence of unsaturated hydroxylated aldehydes of general formula $C_nH_{n-2}O_2$. Thus, the peak at m/z 336 could also be the hydrazone of 4-hydroxy nonenal. This possibility has been investigated using tandem mass spectrometry (data not shown). Although the results do not exclude the presence of 4-hydroxynonenal, the fragmentation pattern of the ion of m/z 336 indicates that the predominant species is the saturated aldehyde. In the mass spectra of samples 8-1 and 14-1 we can observe the presence of aldehydes with the general formula of $C_nH_{2n}O$, but we can also observe a second series of ions, with lower abundance, corresponding to unsaturated aldehydes with general formula of $C_nH_{2n-2}O$.

The mass spectra of fractions 8-1 and 14-1 contain essentially the same species, however, their relative abundances are different (Table 1). The relative abundances of the observed hydrazones were normalised using the intensity of the peak of the most abundant hydrazone that corresponded to dodedecanal, of m/z 364. It should be noted that the low-pressure conditions for electron ionisation cause each mixture component to

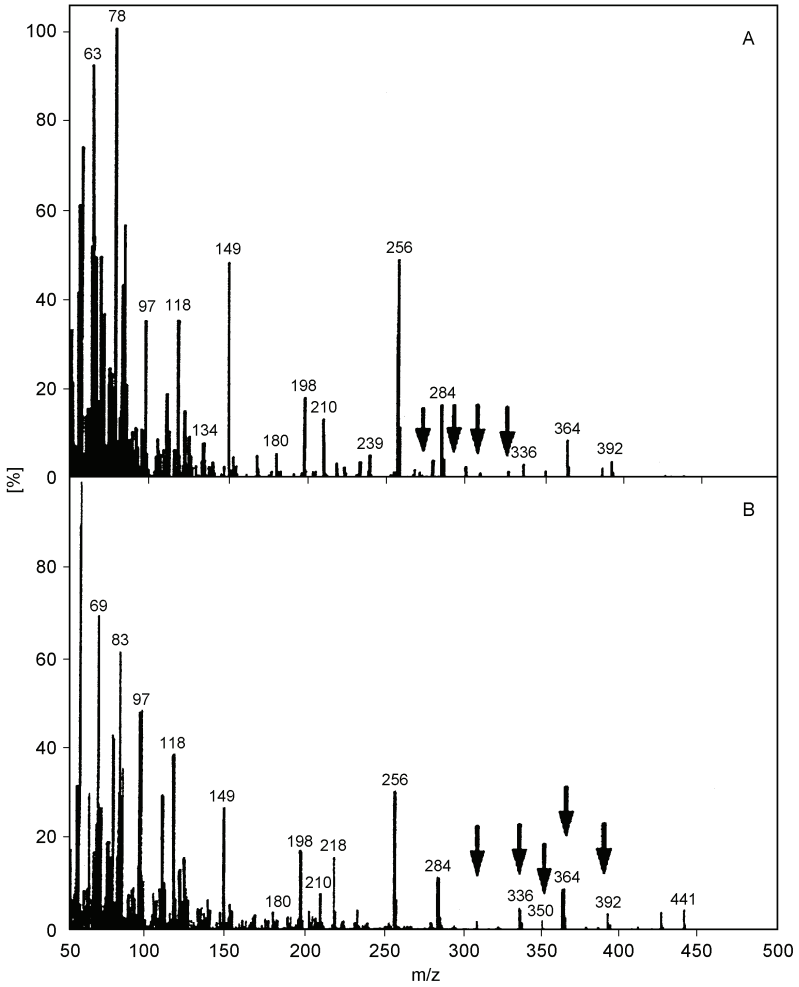

Fig. 3. Typical examples of mass spectrometric analysis of hydrazones of fraction 8-1 (A) and fraction 14-1 (B). The peaks labelled with arrows correspond to the hydrazones with the general formula of the aldehydes $C_nH_{2n}O$. (m - molecular mass, z - charge).

Table 1. Relative abundance in EI mass spectra of the observed hydrazones in the fractions 14-1 and 8-1. They are normalized using the intensity of the peak of the most abundant hydrazone (* - composition of the respective aldehyde that produced the hydrazone of given m/z, n.d. - not detected).

m/z	Corresponding aldehyde*	Relative abundance [%] 14-1	8-1
238	C_3H_6O	11	16
252	C_4H_8O	7	9
266	$C_5H_{10}O$	7	10
280	$C_6H_{12}O$	12	1
294	$C_7H_{14}O$	8	2
308	$C_8H_{16}O$	17	8
322	$C_9H_{18}O$	8	1
336	$C_{10}H_{20}O$	46	36
350	$C_{11}H_{22}O$	20	15
364	$C_{12}H_{24}O$	100	100
378	$C_{13}H_{26}O$	7	4
392	$C_{14}H_{28}O$	45	49
406	$C_{15}H_{30}O$	3	n.d.
420	$C_{16}H_{32}O$	5	20

contribute independently to the measured spectrum of the sample (McLafferty *et al.* 1999). Thus young and old cotyledons differ both in quantity, and aldehyde composition.

Analyses of the hydrazones chromatographic fractions by mass spectrometry revealed differences in abundances of individual aldehydes between young and old cotyledons. In both cases the most abundant species was a C12 aldehyde. There are several indications for the occurrence of unsaturated aldehydes. However, these would be present in much lower abundancies. This observation indicates differences in the products of membrane lipid peroxidation in plants and animals, where the major aldehyde represents 4-hydroxy nonenal (Tamura and Shibamoto 1991).

The ageing of plant organs is accompanied by an increased generation of free radicals similarly as in animals. This study showed that non-polar aldehydes, the typical products of membrane lipid peroxidation, increased during cotyledon ageing. We have observed certain aldehydes to increase specifically in the old cotyledons. In parallel, fluorescent end-products of lipid peroxidation increased in the old cotyledons. The difference of the HPLC fractionation pattern between LFP from the old and young cotyledons might reveal the specificity in nonpolar aldehydes production during cotyledon ageing. In order to ascribe certain aldehydes to a specific LFP fraction it would be necessary to determine their structure in greater detail in future studies.

References

Beckman, J.K., Morley, S.A., Jr., Greene, H.L.: Analysis of aldehydic lipid peroxidation products by TLC/densitometry. - Lipids **26**: 155-161, 1991.

Bolgar, M.S., Gaskell, S.J.: Determination of the sites of 4-hydroxy-2-nonenal adduction to protein by electrospray tandem mass spectrometry. - Anal. Chem. **68**: 2325-2330, 1996.

Chio, S., Tappel, A.L.: Synthesis and characterization of the fluorescent products. - Biochemistry **8**: 2821-2827, 1969.

Chowdhury, P.K., Halder, M., Choudhury, P.K., Kraus, G.A., Desai, M.J., Armstrong, D.W., Casey, T.A., Rasmussen, M.A., Petrich, J.W.: Generation of fluorescent adduts of malondialdehyde and amino acids: Toward an understanding of lipofuscin. - Photochem. Photobiol. **79**: 21-25, 2004.

Dhindsa, R.J., Dhindsa, P.P., Thorpe, T.A.: Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation and decreased levels of superoxide dismutase and catalase. - J. exp. Bot. **32**: 93-101, 1981.

Esterbauer, H., Koller, E., Slee, R.G., Koster, J.F.: Possible involvement of the lipid-peroxidation product 4-hydroxynonenal in the formation of fluorescent chromolipids. - Biochem. J. **239**: 405-409, 1986.

Gerstbrein, B., Stamatas, G., Kollias, N., Driscoll, M.: *In vivo* spectrofluorimetry reveals endogenous biomarkers that report healthspan and dietary restriction in *Caenorhabditis elegans*. - Aging Cell **4**: 127-137, 2005.

Hudak, K., Yao, K., Thompson, J.E.: Release of fluorescent peroxidized lipids from membranes in senescing tissue by blebbing of lipid-protein particles. - Hort. Sci. **30**: 209-213, 1995.

Kikugawa, K., Machida, Y., Kida, M., Kurechi, T.: Studies on peroxidized lipid. III. Fluorescent pigments derived from the reaction of malonaldehyde and amino acids. - Chem. Pharm. Bull. **29**: 3003-3011, 1981.

Maguire, Y.P., Haard, N.F.: Fluorescent product accumulation in ripening fruit. - Nature **258**: 599-600, 1975.

McLafferty, F.W., Stauffer, D.A., Loh, S.Y., Wesdemiotis, C.: Unknown identification using reference mass spectra. Quality evaluation databases. - J. amer. Soc. Mass Spectrometry **10**: 1229-1240, 1999.

Merzlyak, M.N., Plakunova, O.V., Gostimsky, S.A., Rumyantseva, V.B., Novak, K.: Lipid peroxidation in and photo-damage to a light-sensitive chlorescence pea mutant. - Physiol. Plant. **62**: 329-334, 1984.

Pauls, K.P., Thompson, J.E.: Evidence for the accumulation of peroxidized lipids in membranes of senescing cotyledons. - Plant Physiol. **75**: 1152-1157, 1984.

Procházková, D., Wilhelmová, N.: Changes in antioxidative protection in bean cotyledons during natural and continuous irradiation-accelerated senescence. - Biol. Plant. **48**: 33-39, 2004.

Sohal, R.S., Allen, R.G.: Relationship between oxygen metabolism, aging and development. - In: Sohal, R.S. (ed.): Advances in Free Radical Biology and Medicine.Vol. 2. Pp. 117-159. Pergamon Press, New York 1986.

Tamura, H., Shibamoto, T.: Gas chromatographic analysis of malonaldehyde and 4-hydroxy-2-(E)-nonenal produced from arachidonic acid and linoleic acid in a lipid peroxidation model system. - Lipids **26**: 170-173, 1991.

Wang, W., Sun, G.-C., Lin, Z.-F.: Lipofuscin-like pigments in chloroplasts and effects of malonaldehyde and alpha-

tocopherol on its accumulation. - Plant. Physiol. Commun. **3**: 31-34, 1988.

Wilhelm, J., Wilhelmová, N.: Accumulation of lipofuscin-like pigments in chloroplasts from senescent leaves of *Phaseolus vulgaris*. - Photosynthetica **15**: 55-60, 1981.

Wilhelmová, N., Procházková, D., Macháčková, I., Vágner, M., Srbová, M., Wilhelm, J.: The role of cytokinins and ethylene in bean cotyledon senescence. The effect of free radicals. - Biol. Plant. **48**: 523-529, 2004.

Wilhelmová, N., Wilhelm, J., Kutík, J., Haisel, D.: Changes in French bean cotyledon composition associated with modulated life-span. - Photosynthetica **34**: 377-391, 1997.

Yao, K., Paliath, G., Thompson, J.E.: Localized of peroxidized lipids in nonsedimentable microvesicles of senescing bran cotyledons. - J. exp. Bot. **44**: 1267-1274, 1993.

Yin, D.: Biochemical basis of lipofuscin, ceroid, and age pigment-like fluorophores. - Free Radical Biol. Med. **21**: 871-888, 1996.