Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 24, issue 4, 2014, pp.413 - 418

CHLOROPHYLL CATABOLISM IN DARKENED HAMAMELIDACEAE LEAVES

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ABSTRACT. Chlorophyll catabolism was induced in two Hamamelidaceae plant species by placing detached leaves in darkness. The induced chlorophyll catabolism was observed by visible leaves' yellowing. The extracts of the yellowed leaves were analyzed by liquid chromatography-mass spectrometry. On the basis of the chlorophyll catabolites registered in the chromatograms, summarized were the results which permitted the proposition of the chlorophyll catabolic pathway in two Hamamelidaceae plant species under dark-induced senescence. The time interval for the induction of chlorophyll catabolism, in two Hamamelidaceae plant species, differed significantly.

Keywords: chlorophyll catabolism, dark-induced senescence, Hamamelidaceae

INTRODUCTION

In autumn, the most observable biochemical process is chlorophyll biodegradation. Chlorophylls are catabolized and visible are flavonoids, anthocyanin, caretonoid and other plant pigments. Chlorophyll catabolism proceeds with the hydrolysis of chlorophyll *a* to give chlorophyllide *a*. After the release of magnesium from the core of chlorophyllide *a*, pheophorbide *a* is formed. Oxygenolitic cleavage of pheophorbide *a*, at the "north" methane bridge, gives a tetrapyrrole. The subsequent oxidation reactions induce further modifications on the tetrapyrrole side chain groups (Djapic, 2013).

The tetrapyrrole's ethyl side chain is then hydroxylated. The enzyme catalyzing the hydroxylation of the ethyl side chain is still unknown. It was reported that in tobacco leaves, the activity of peroxidase was the indicator of senescence (Parish, 1968). Peroxidase activity, during leaf senescence, was, also, registered in: bean, sunflower and rice leaves (Braber, 1980; Hazell and Murray, 1982; Kar and Mishra, 1976). In the next step of chlorophyll catabolism in Hamamelidaceae plant species, the aldehyde group of the tetrapyrrole with hydroxylated ethyl side chain is oxidized. The structure of the tetrapyrrole formed refers to urobilinogen (Djapic, 2014a). The enzyme catalyzing the oxidation of the aldehyde group is still unknown. The proposed mechanism can be the oxidation with Bayer-Villiger-ase. It can be proposed that this enzyme converts the aldehyde group of the tetrapyrrole with the hydroxylated ethyl side chain into the corresponding dehydro pyrrolidin-2-one mediated by hydroperoxide intermediate. This mechanism compromises the nucleophilic addition of hydroperoxide to the oxo group affording peroxide intermediate. After the rearrangement, the insertion of oxygen is made. After the hydrolysis and tautomerization the tetrapyrrole, with the structure that refers to urobilinogen, is formed. The other mechanism that can explain the formation of the tetrapyrrole, with the structure that refers to urobilinogen, is by oxidative decarboxylation of an acid intermediate after the oxidation of the aldehyde group. Next mechanism that can be proposed is the reaction with the hydroxy-radical.

Under acidic conditions, the tautomerization is initialized inducing the formation of the thermodynamically more stable tetrapyrrole's ring D. After the tautomerization, the subsequent reduction of one tetrapyrrole methene bridge induces the loss of the fluorescence (Djapic, 2012).

Leaf senescence can be induced by exogenous factors. Darkness is one of the inducers of senescence. It was shown that adult attached leaves do not induce senescence when the whole plant is placed in darkness and senescence was induced in detached leaves placed in darkness (Weaver and Amasino, 2001).

It is reported that the degradative processes, such as decay of chlorophyll and the onset of proteolysis are more rapid in darkness (Martin and Thimann, 1972; Trippi and DeLuca D'Oro, 1985; Kanazawa *et al.*, 2000). Change in membrane characteristics, mainly caused by free radical-induced lipid peroxidation, is another visible change connected with senescence in plants, both in light and in darkness. Radical species formed from molecular oxygen as well as singlet oxygen are responsible for the oxidative processes which can damage lipids, chlorophylls and proteins (Borraccino *et al.*, 1994). The stress-related phenomena: wounding, drought and salinity, both in plant organs and in isolated tissues and cells induce chlorophyll catabolism.

In order to determine whether the detached leaves of two plants species from the Hamamelidaceae plant family have the same chlorophyll catabolism as autumnal leaves the following experiment was carried out.

MATERIALS AND METHODS:

Two Hamamelidaceae plant species: *Parrotia persica* (*Pp*) and *Hamamelidaceae virginiana* (*Hvir*) branches with green leaves were cut in summer, placed in 1000 ml beakers with demineralized water and placed in complete darkness. The visible yellowing of *Pp* occurred after a month and in *Hvir* after two weeks. Yellow leaves were extracted with methanol, at room temperature. After centrifugation, methanol extract was filtered and partitioned between hexane and methanol. Water was added to the methanol phase. Chlorophyll catabolites were extracted with dichloromethane from

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Article published: November 2014

the methanol-aqueous phase. Dichloromethane was evaporated (t<40ºC). The extracts obtained were dissolved in 1.5 ml methanol – water (2:1) mixture and subjected to the liquid chromatography mass spectrometry (LC-MS) analysis. Methanol and water used for the LC separation were HPLC grade (Acros Organics, Geel, Belgium) and trifluoroacetic acid (TFA) was reagent grade (Fluka, Buch, Switzerland). The LC/UV/ electrospray ionization (ESI) – MS analysis were performed on Waters 2695 Separations Module (Milford, MA, USA) coupled to a Waters 2996 photodiode array (PDA) UV-Vis detector and connected to Bruker Daltonics esquire high capacity ion trap (HCT) (Bruker Daltonik, GmbH, Bremen, Germany) equipped with an electrospray ionization (ESI) source. Nitrogen produced by nitrogen generator (Domnick Hunter Group plc, Durham, England) was used as nebulizer (20 psi) and drying gas (9 L min^{-1} at 320ºC) in the ESI experiments. The ESI detection was done in the positive ion mode with the target mass of 900 *m*/*z*. The capillary voltage in a ramp ranged from 4.5 to 1.5 kV. Data were acquired by HyStar™ and processed by Bruker Daltonics Data Analysis running under Windows NT™ (Microsoft, Redmond, USA). LC separation was carried on reverse phase (RP) EC 250x4 mm Nucleosil 100-5 C8 column together with RP CC 8x4 mm Nucleosil 100-5 C8 precolumn (Macherey-Nagel, Oesingen, Switzerland). The temperature of the column oven was 22ºC for analytic measurements. The injection volume was 10 µl via autosampler injection. Mobile phase consisted of 0.1% TFA (modifier) in water and methanol. The proportion of methanol was increased linearly from 10% to 100% in 70 minutes and in next 20 minutes elution was continued with methanol. The flow rate of 0.2 ml/min. was used for LC/MS chromatographic method. After each separation the column was reequlibrated linearly from 100% methanol to 90% water (0.1% TFA):10% methanol in 10 minutes and additional 5 minutes at 90% water (0.1% TFA):10% methanol. Data were acquired by HyStar™ and processed by Bruker Daltonics Data Analysis running under Windows NT™ (Microsoft, Redmond, USA).

Extracts' purification was done by MPLC, on lab – made column, size 310 x 25 mm, filled with LiChroprep® RP8, particle size 25-40µm silica gel (Merck, Darmstadt, Germany). All solvents used for MPLC were distilled prior to use. Elution with water: methanol, 1:1→0:1 in 30 min. (flow rate 7 ml/min; UV detection 280 nm) yielded the prepurified chlorophyll catabolites. Final purification was done by RP HPLC using Waters 600 HPLC system coupled with Waters 2996 PDA UV-Vis detector (Waters Corp., Milford, USA) and RP EP 250x16 mm Nucleosil 100-7 C_8 column together with the RP EP 30x16 mm Nucleosil 100-7 C_8 precolumn (Macherey-Nagel, Oesingen, Switzerland). The detection wavelength was set at 244 nm, temperature of the column oven was 22^0 C and the injection volume was 2 ml via loop injection. The mobile phase consisted of water (0.1%TFA): methanol, 1:1 (v/v) and ran isocratically at a constant flow rate of 3.2 ml/min for chlorophyll catabolites **6**. For other chlorophyll catabolites the mobile phase consisted of water (0.1%TFA): methanol, 1:3 (v/v) and ran isocratically at a constant flow rate of 3.2 ml/min. PDA detection was in range of 200-800 nm, chromatogram was extracted at λ=244 nm. All solvents used were HPLC grade (Acros Organics, Geel, Belgium).

^{1}H NMR spectra were recorded on a Bruker Avance DPX 360 NMR spectrometer. Chemical shifts are relative to TMS at δ 0.00, but were referenced to residual proto – deutero solvent signals in CD_3OD.

RESULTS AND DISCUSSION:

Detached branches with green leaves of the two Hamamelidaceae plant species *Pp* and *Hvir* were placed in complete darkness. After two weeks in complete darkness, the *Hvir* yellow leaves were extracted and subjected to LC-MS analysis. In case of *Pp*, the yellow leaves were extracted and subjected to LC-MS analysis after a month in complete darkness (Fig. 1).

Fig. 1. *Pp* detached branches, after cutting and placing them in a beaker (left) and after a month spent in complete darkness (right)

The LC – MS analysis of the *Pp* and *Hvir* extracts from dark-induced senescence leaves were subjected on the RP – C_8 analytical column. Chlorophyll catabolites depicted in Figure 2 were present in chromatograms obtained (Fig. 3).

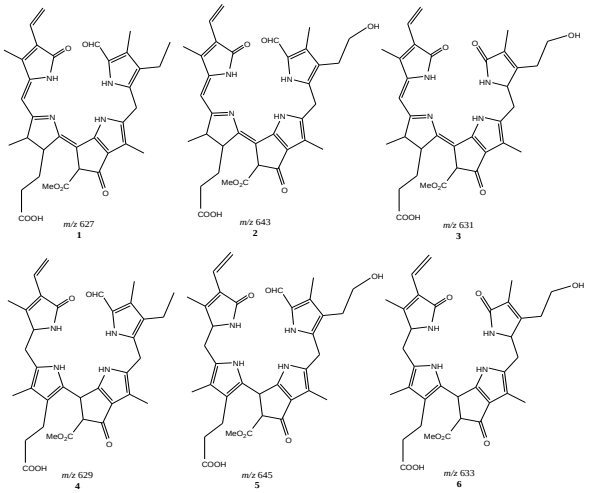


Fig. 2. Structure of chlorophyll catabolites present in dark-induced *Pp* and *Hvir* leaves

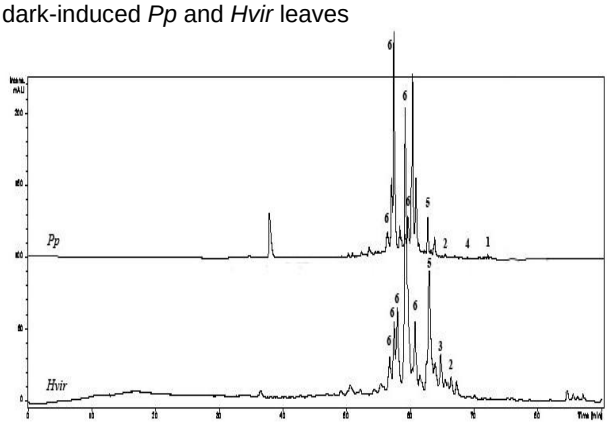
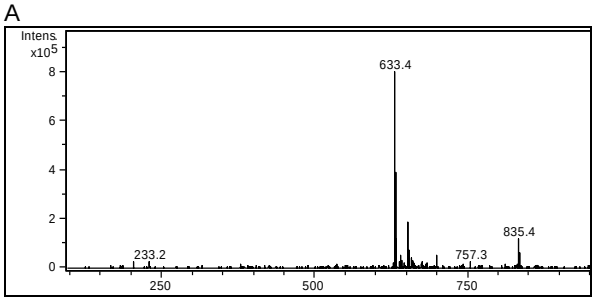


Fig. 3. Chromatograms of *Pp* and *Hvir* extracts from dark-induced senescence leaves

Separation conditions: mobile phase: water (0.1% TFA): methanol, gradient elution, column temperature 22ºC, flow rate 0.2 ml/min. Detection: UV 244 nm, injection: 10 µl, on RP C-8 column.

In *Pp* extract present were 3 isomers of the chlorophyll catabolite **6** and chlorophyll catabolites **1**, **2**, **4** and **5**. In *Hvir* extract present were five isomers of the chlorophyll catabolite **6** and chlorophyll catabolites **2**, **3** and **5**. As part of chlorophyll catabolism studied in Hamamelidaceae leaves, liquid chromatography-electrospray-tandem mass spectrometry (LC-ESI-MS) used detected known chlorophyll catabolites in dark induced senescence leaves' extracts. Chromatogram of *Hvir* extract revealed the presence of chlorophyll catabolite **6** at retention time 59.3 min. and four its isomers at retention time: 56.9, 57.5, 58.1 and 60.9 min. (Fig. 3 and 4). Ion mass spectra of chlorophyll catabolite **3** with *m/z* 631 and **5** with *m/z* 645 are depicted in Fig. 5.



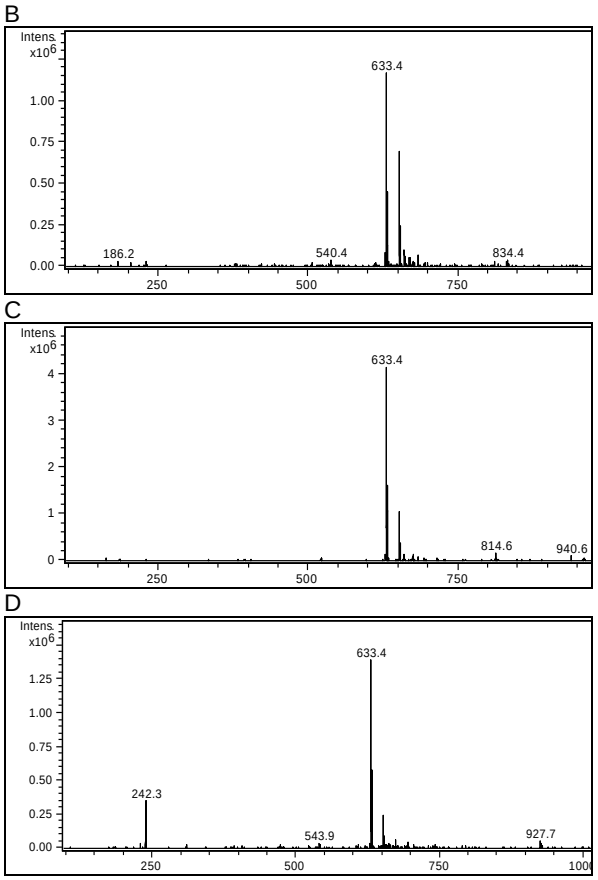


Fig. 4. Ion mass spectra of chlorophyll catabolite **6** and its isomers in *Hvir* extracts from dark-induced senescence leaves. The retention time for A, B, C and D were: 57.5, 58.1, 59.3 and 60.9, respectively

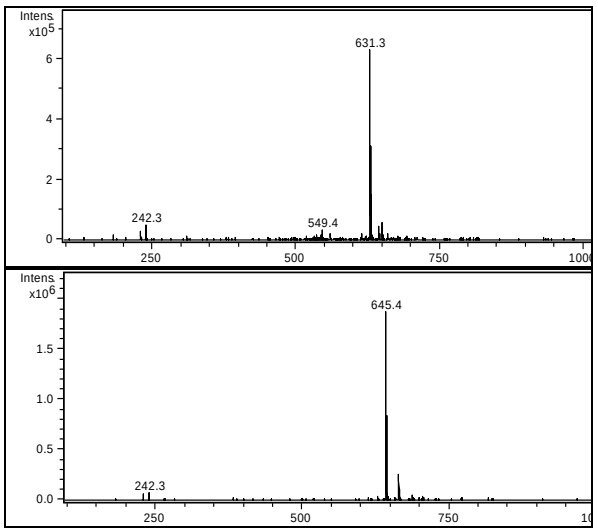


Fig. 5. Ion mass spectra of chlorophyll catabolite **3** (left) and **5** (right). The retention time for **3** was 64.9 min. and for **5** was 63.0 min.

Extracts of dark-induced senescence Hamamelidaceae leaves were purified on RP MPLC and final purification was performed on semi-preparative RP HPLC. Proton spectra of chlorophyll catabolite **5** and of two isomers of chlorophyll catabolite **6** are depicted in Fig. 6, 7 and 8. In tetrapyrrole chlorophyll catabolites' proton spectra signals can be subdivided into several regions. In the high magnetic field region four signals of methyl group

appear. Chemical shifts of propionyl side chain protons, next to the carboxyl group and ethylene hydroxyl side chain H_A proton next to the pyrrole ring B resonate at 2.3-2.5 ppm. The methylene H_A protons bridging the pyrrole rings fall in the spectral region of 2.5 ppm along with the resonance of propionyl side chain protons next to the pyrrole ring D. The H_B protons of ethylene hydroxyl side chain, next to the pyrrole ring B, and H_B protons of methylene atoms bridging the pyrroles resonate in the region from 2.8 to 3.2 ppm. Singlet at 3.35 ppm indicates the identity of five membered ring carboxymethyl side chain protons. The residual water signal (HDO signal) is the most abundant one. Protons within five-membered ring system and protons on the pyrrole A and B ring resonate in the region 3.75-4.4 ppm. Vinyl protons are found in the low magnetic field region and can be assigned by their characteristic chemical shifts and coupling constants. The formyl group proton on the pyrrole ring B, in case of the chlorophyll catabolite **5**, resonates in the low field at 9.5 ppm. Chlorophyll catabolite isomers **6** lack this low field signals. Deutero-exchange occurs on heteroatoms bearing protons within 12 hours.

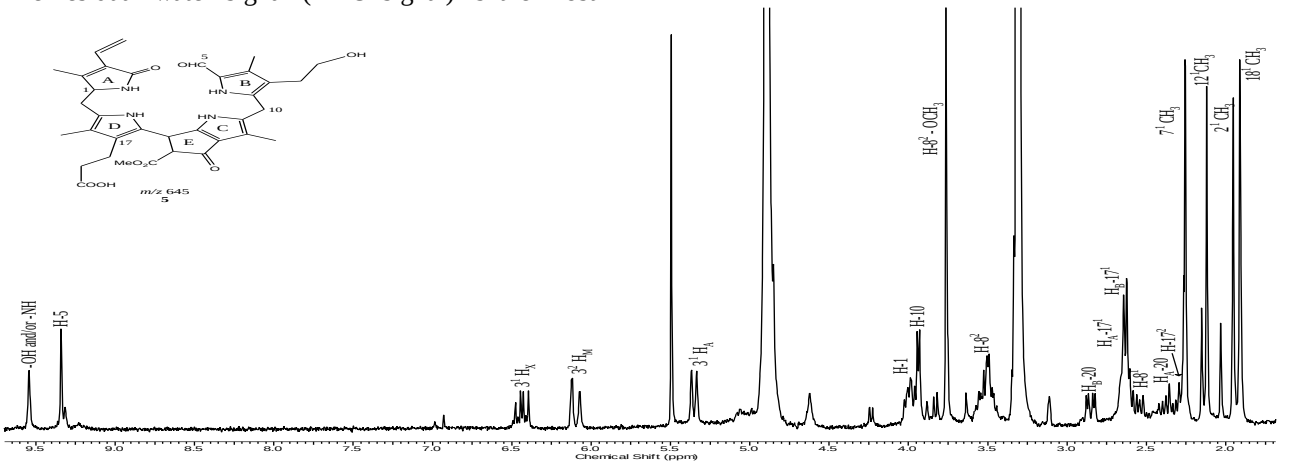


Fig. 6. Proton spectrum of chlorophyll catabolite **5**

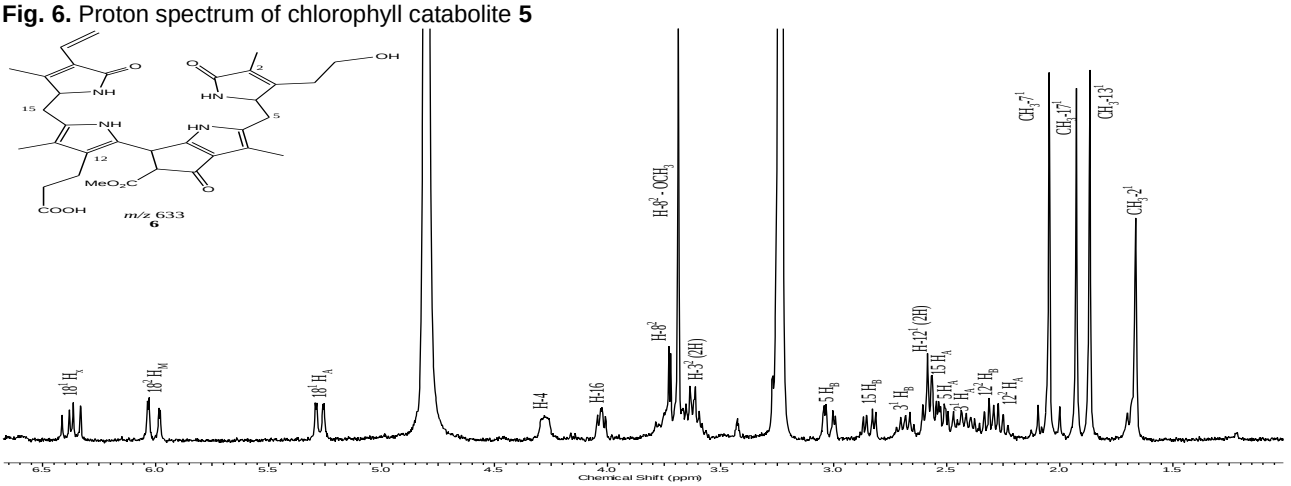


Fig. 7. Proton spectrum of the chlorophyll catabolite **6** (the most abundant isomer in *Pp* extract from dark-induced senescence leaves)

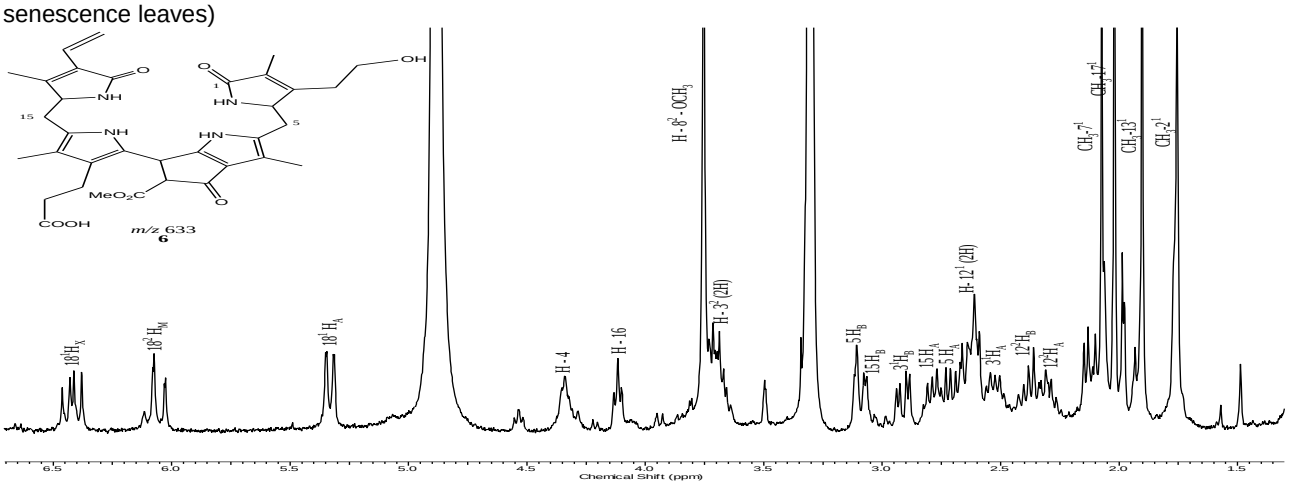


Fig. 8. Proton spectrum of the chlorophyll catabolite **6** (the most abundant isomer in *Hvir* extract from dark-induced senescence leaves)

Leaves in which senescence was induced by placing the green mature leaves on branches in total darkness gave insight in chlorophyll catabolism. The chlorophyll catabolic pathway that can be proposed is depicted in Fig. 9. For the time being, all isomers will be placed in one chlorophyll catabolic step, according to Dirichlet's box principle (Anderson, 2003). Chlorophyll catabolism, in Hamamelidaceae plant species, after opening of the porphyrin ring follows the next chlorophyll catabolic steps: chlorophyll catabolite **1** is hydroxylated at the $C8^2$ position to form the hydroxylated ethyl side chain chlorophyll catabolite **2**.

The chlorophyll catabolite **2** undergoes the oxidation of the aldehyde group and the tetrapyrrole formed refers to urobilinogen **3**. Under acidic conditions chlorophyll catabolites **1**, **2** and **3** tautomerize and thermodynamically more stable catabolites are formed (Djapic, 2012). The reduction of the thermodynamically more stable chlorophyll catabolites, at the "western" methene bridge, in the presence of ferredoxin, gives chlorophyll catabolites **4**, **5**, **6** (Fig. 10). Isolation of further chlorophyll catabolites will enable the switch to the Theory of parallel lines (Lobatschewski, 1985; Popov, 2010; Чистяков, 1973).

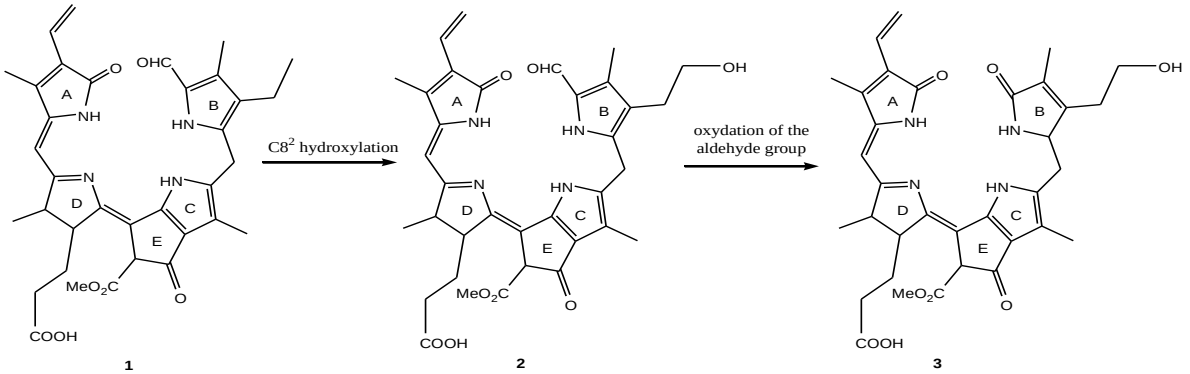


Fig. 9. Chlorophyll catabolic pathway in dark-induced *Pp* and *Hvir* leaves

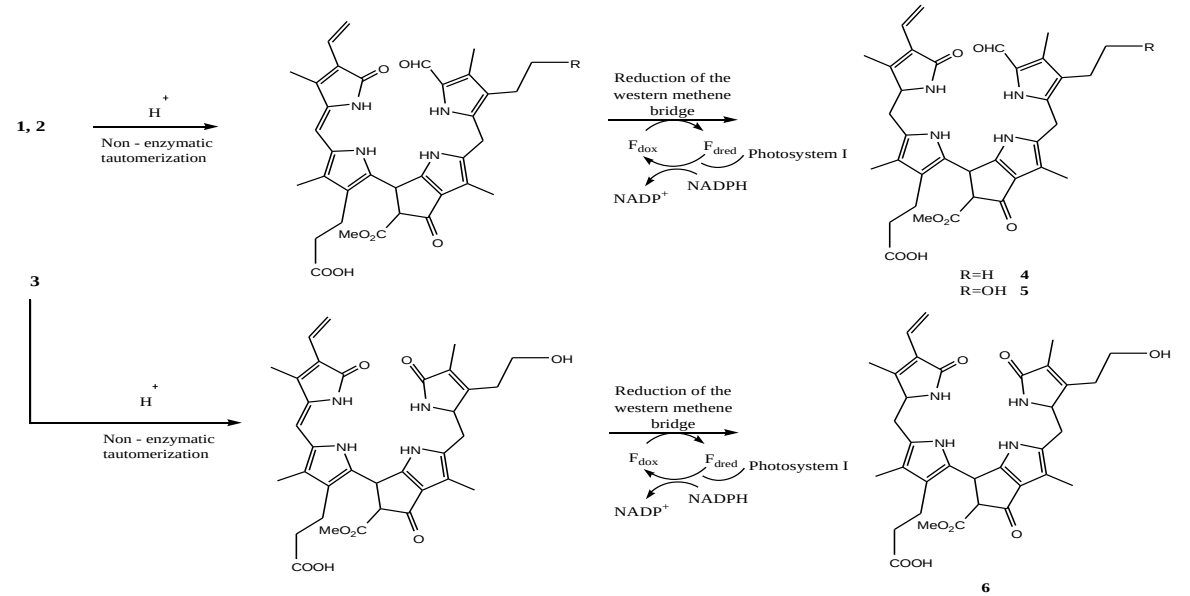


Fig. 10. Tautomerization under acidic conditions and reduction of the formed thermodynamically more stable chlorophyll catabolites

CONCLUSIONS:

Chlorophyll catabolism in autumnal and dark-induced senescence leaves has the same chlorophyll catabolitic pathway (Djapic, 2014a, 2014b). That can be judged by chlorophyll catabolites present in the analyzed samples of autumnal and dark-induced senescence leaves' extracts. Hamamelidaceae plants have the same mechanism for chlorophyll catabolism under natural conditions and under stress conditions. Natural sciences are based on the uniform law of cause and effect (Harrington, 2001). In this case, the cause is darkness and induced effect is yellowing of leaves, for both plant species analyzed. In case of *Hvir* the effect-yellowing of leaves and chlorophyll catabolism occurs two weeks ahead from *Pp* leaves, on the detached branches left in permanent darkness. It can be postulated that the cause, in this case darkness, induces a signal or signals beginning the chlorophyll catabolism. Not taking into account, what the signal is or what the signals are, it can be postulated that the signal is or signals are, at least two times more rapid and stronger in *Hvir* leaves than in *Pp* leaves.

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