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Chapter 10

EPR Spectroscopy — A Valuable Tool to Study Photosynthesizing Organisms Exposed to Abiotic Stresses

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Additional information is available at the end of the chapter

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1. Introduction

Abiotic environmental stresses, such as heat, cold, salt, drought, excess of photochemically active radiation (PAR) as well as UV-A and UV-B radiation, or presence of gaseous pollutants (e.g. ozone or SO_2), heavy metals or herbicides in the environment lead to inhibition of photosynthetic processes. Some of these abiotic stresses target specific cellular pathways, other ones have a broad cellular impact. They adversely affect photosynthetic apparatus of photosynthesizing organisms what ultimately results in negative effects on plant growth, productivity in agriculture, metabolic profile as well as plant nutritional potential. Therefore, plant abiotic stress has been a matter of concern for the maintenance of human life on earth and especially for the world economy [1].

The great power of EPR is its ability to identify the chemical nature of free radical species, and from the intensity of the signal to determine the number of radicals that have been formed in particular systems. Many components of the photosynthetic apparatus provide EPR signals at certain conditions (in detail see in subchapter 3). From the line widths and line shapes of the EPR spectra of radical species, frequently deliberately introduced to samples as spin-probes, various features of the local molecular environment may further be deduced [2].

Functional and undamaged thylakoid membrane is essential for successful process of photosynthesis. Based on EPR measurements using spin probes which are suitable to evaluate the effects of membrane-active compounds on the fluidity of PS 2 membranes or to study the fluidity of chloroplast thylakoid membranes of plants exposed to stressful conditions (e.g. herbicides, frost, etc.) changes in rotational mobility of lipids could be determined [3,4]. From the changes in EPR spectra relative membrane perturbation could be evaluated. On the other

hand, rotational correlation time values (τ_c) can be used to monitor the effect of membrane active compounds on the rate of molecular reorientation of spin label in order to determine changes in the microviscosity of thylakoid membranes caused by these compounds [3].

Exposure of photosynthesizing organisms to stressful conditions such as drought, salinity, low temperature or heavy metals is connected with increased production of reactive oxygen species (ROS) that are generated due to the stepwise reduction of molecular oxygen by high-energy exposure or as a result of electron transfer chemical reactions. The enhanced production of ROS, including free radicals such as superoxide anion ($O_2^{-\bullet}$) and hydroxyl radical ($^\bullet$OH), as well as non-radical molecules H_2O_2, ozone and singlet oxygen ($^1O_2$), which occur during abiotic stresses, results in lipid peroxidation and oxidative damage of proteins, nucleic acids as well as in inhibition of enzyme activity [5]. EPR spectroscopy is a suitable method for qualitative and quantitative evaluation of ROS in photosynthesizing organisms. Experimental evidence of ROS generation in photosystem 2 particles can be directly obtained by EPR technique in combination with suitable spin trap after their irradiation by visible light [6]. This technique is also suitable to determine deleterious effects of UV-B and UV-A radiation on the photosynthetic apparatus [7-9].

2. Light reactions of photosynthesis

Photosynthesis is a process in which plants convert solar energy into chemical energy. In light reactions of photosynthesis the green algae and higher plants use two different reaction centres, called photosystem (PS) 1 and 2, while purple bacteria make do with a single reaction centre. The process of photosynthesis starts by the capture of photons in the antenna system of pigment-protein complexes and subsequent formation of excited forms of pigments. Excitation energy is then transported from the antenna to the cores of both photosystems where the primary charge separation occurs. Subsequent electron transfer steps prevent the primary charge separation from recombining by transferring the electron through the photosynthetic electron transport (PET) chain by a system of suitable electron acceptors and electron donors. The current concept of electron transport in the photosynthetic apparatus of green algae and higher plants is reflected in the following scheme:

$$H_2O \rightarrow OEC \rightarrow Z\,/\,D \rightarrow P680 \rightarrow Pheo \rightarrow Q_A \rightarrow Q_B \rightarrow PQ \rightarrow Fe_2S_2 \rightarrow Cytf \rightarrow$$
$$\rightarrow PC \rightarrow P700 \rightarrow A_0 \rightarrow A_1 \rightarrow F_X \rightarrow F_A \rightarrow F_B \rightarrow F_d \rightarrow NADP^+$$

where OEC is oxygen evolving complex; Z/D are intermediates which participate at electron transport from OEC to P680, which is the core of PS 2 consisting from chlorophyll (Chl) *a* dimer; Pheo is pheophytin, the first electron acceptor in PS 2; Q_A and Q_B are the first and the second quinone acceptors of electron; PQ is plastoquinone pool consisting of a set of quinones; Fe_2S_2 is Rieske iron sulphur protein complex; cyt *f* is cytochrome *f*; PC is plastocyanin; P700 is the core of PS 1 consisting of Chl*a* dimer; A_0, a Chl*a* molecule, represents the primary electron acceptor in PS 1; A_1, a phylloquinone, is the secondary electron acceptor; F_X, F_A and F_B are the iron-sulphur centres; F_d is ferredoxin and $NADP^+$ is the final electron acceptor of PS 1 [10,11].

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Photosystem 2 is the only known protein complex that can oxidize water, which results in the release of O_2 into the atmosphere. The catalytic cleaving of water occurs at a cluster consisting of four manganese atoms and one calcium atom which is situated at the luminal side of two key polypeptides of PS 2, D_1 and $D_{2,}$. Water oxidation requires two molecules of water and involves four sequential turnovers of the reaction centre and manganese in the cluster undergoes light-induced oxidation. A kinetic model of oxygen evolution based on five S-states which was developed by Kok and co-workers postulated that each photochemical reaction creates an oxidant that removes one electron, driving the oxygen evolving complex to the next higher S-state [12]. The result is the creation of four oxidizing equivalents in the oxygen evolving complex. Electrons which were formed during photochemical cleavage of water are then transferred to P680 via Tyr_Z, a redox-active tyrosine residue on D_1 protein. From P680 the electrons are then transported to a mobile pool of plastoquinone molecules by subsequent redox reactions via Pheo, Q_A and Q_B. The electrons are further transmitted to the cytochrome complex and to the PS 1. The final electron acceptor of PS 1 is $NADP^+$ [10].

3. EPR signals of components of photosynthetic apparatus

The method of electron paramagnetic (spin) resonance (EPR or ESR) is suitable for detection of compounds which contain unpaired spins. During electron transport through the photosynthetic apparatus radicals are formed which can be recorded by EPR spectroscopy.

In the manganese cluster of OEC four manganese atoms, occurring in oxidation states II, III and IV, are bound to a 33 kDa protein. Due to spin-spin interactions the bound manganese atoms show no EPR signals at room temperature. After irradiance with light impulses four different states of OEC, in the literature known as S_0 to S_3, can be registered. In PS 2 particles prepared from spinach chloroplasts at cryogenic temperatures EPR spectra of some S states could be recorded.

The state S_0 consists of two signals. The first is similar to the multiline signal which is centred near g = 2.0 and spread over ~ 238 mT. It is constituted of 25 resolved lines spaced in the region of 6.5 – 9.5 mT. This signal was assigned to the antiferro-magnetically coupled S = 1/2 system in the ground state. The second signal is broad featureless signal at low field with g = 6 and g = 10 (S = 5/2) [13,14,15,16]. The integer spin of S_1 state (S = 1) exhibits two low field signals which were recorded by parallel-polarization mode EPR spectrometer. They are situated in low field region, the first at g = 12 with 18 lines (spacing 3.2 mT) and the second one at g = 4.8 (width 60 mT) [14,15,16]. S_1 state also exhibits a multiline EPR signal (S = 1/2) at g = 2.0 which is attributed to $S_1Q_A^-Fe^{2+}$ state [17] or to $S_1Y_Z^\bullet$ state [18]. Two EPR signals have been attributed to the manganese complex in the S_2 state. These signals are observable only at temperatures < 35 K. One of these signals is the multiline signal, ascribed to the ground state of S = 1/2, centred at g = 1.982 with a line width of 150-180 mT. The first signal consists of 18-20 partially resolved hyperfine lines (distance from each other 8-9 mT) which are often superimposed on broad Gaussian-shaped signal near g = 2. The other signal occurs at g = 4.1 (width ~ 36 mT, S = 5/2) [19,20]. The multiline EPR signal of $S_2Q_A^-Fe^{2+}$ state was also observed in PS 2 membranes [17].

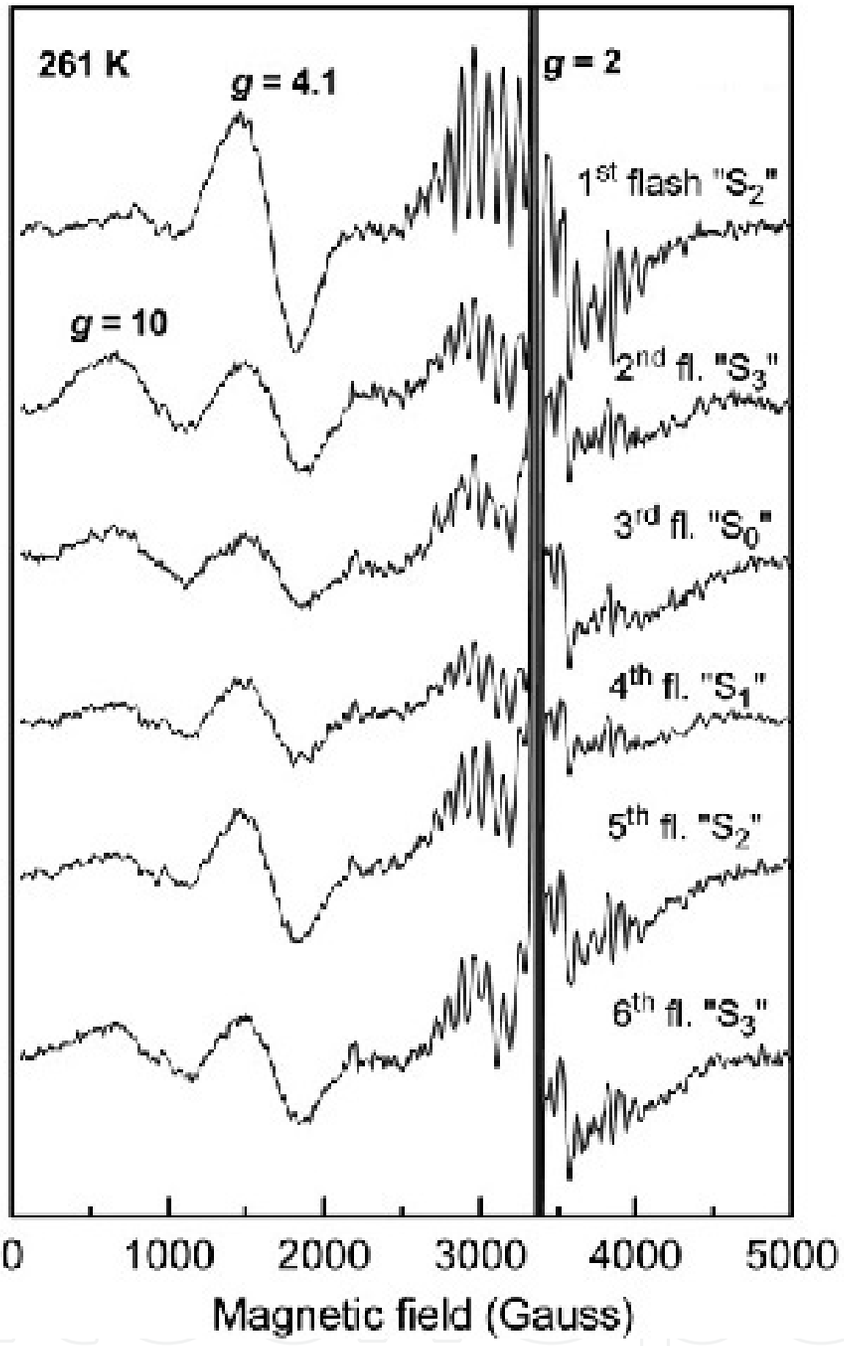

Figure 1. The EPR spectra of PS 2 particles during sequential flash illumination. The sample received each flash at 261 K, followed by rapid cooling to liquid helium temperatures to record the EPR spectrum. Source: [24].

Wider (~ 80 mT) EPR signal S_3 for S = 1/2 in Ca^{2+}-depleted PS 2 membranes was observed in the region of g ~2.0 [21]. The parallel polarization EPR method has been applied to investigate the manganese EPR signal of native S_3 state of OEC in PS 2 with S = 1, g = 11 and g = 15 with a width of 20 - 30 mT [15]. The EPR signal of S_3-state was also observed in the Boussac Works [20,22]. On the other hand, Hallahan et al. [23] assumed that the S_3 signal arises from the S_2-Y_Z^+ interaction. The typical EPR spectra of S states are presented in Fig. 1.

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In EPR spectra of chloroplasts treated with some inhibitors of photosynthesis six line spectrum (at g ~ 2.0) originating from Mn^{2+} ions released from manganese cluster into interior of thylakoid membranes can be observed [25] (Fig. 2B).

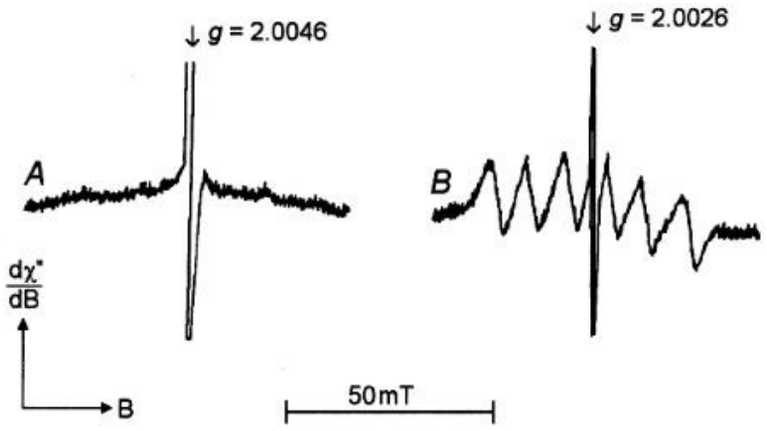

Figure 2. EPR spectra of spinach chloroplasts: A - control sample, B - chloroplasts treated with 0.05 mol dm^{-3} of $HgCl_2$. Source: [26].

EPR signals belonging to Z/D intermediates were observed for the first time in spinach chloroplasts already in 1956 [27]. They are long living radicals which could be recorded at room temperature by continual wave (cw) EPR spectrometer. These signals are situated in the region of free radicals and they are referred in the literature as signal II_{slow} (Fig. 3A, full line) with g = 2.0046 and ΔB_{PP} = 1.9 mT [28] and signal $II_{very\ fast}$ (Fig. 3A, dotted line) with g = 2.0046 and ΔB_{PP} = 1.9 mT [29]. These signals were associated with some components of PS 2 [30]. Signal II_{slow} can be registered even in darkness, while signal $II_{very\ fast}$ is observable only in the light. At the end of 80 years of the last century it was found that signal II_{slow} corresponds to the oxidized intermediate $D^{\bullet}$, i.e. to the tyrosine radical occurring in the 161st position of D_2 protein on the donor side of PS 2 [31]. The EPR signal $II_{very\ fast}$ is observable as an increase of signal II in the light and it belongs to the intermediate $Z^{\bullet}$, i.e. to the tyrosine radical occurring in the 161st position of D_1 protein [32].

EPR signals originating from P680 can be observed in two states: as oxidized $P680^{+}$ with g = 2.0030 and line width of 0.85 mT [17,33] or as a triplet state of P680 (^{3}P680) [34].

Pheophytin is the first acceptor of electrons on the acceptor side of PS 2. After its reduction a radical with short life time ($Pheo^{-}$) is formed and EPR spectrum of this radical (g = 2.0032, ΔB_{PP} = 1.2 mT) can be recorded at cryogenic temperature by the methods of electron spin polarization [35] or by time resolved EPR spectroscopy [36].

Due to the interaction with paramagnetic iron atom, the EPR spectra of Q_A^{-} and Q_B^{-} are registered as very broad signals at g < 2.0. Q_A in triplet state was also recorded by optically detected magnetic resonance spectroscopy (ODMR) [37]. Q-band spectrometer was used for better resolution of anisotropic g tensor for $Q_A^{-\bullet}$and the estimated values of g-tensor

were g_{xx} = 2.0073, g_{yy} = 2.0054 and g_{zz} = 2.0023 [38]. Moreover, the EPR signal from the radical Q_A^--Fe^{2+} with g = 1.67 and 1.82 was registered by Jegerschöld and Styring [39]. On the other hand, van Mieghem et al. [34] registered this signal with g = 1.9.

The EPR spectrum of oxidized core of PS 1 ($P700^+$) belongs to the first observed EPR signals. Commoner and Heise [27] called it as signal I with g = 2.0026 and line width 0.8 mT. This signal is well visible in Fig. 3C (both lines). Later, Warden and Bolton [40] found that this signal belongs to chlorophyll *a* dimer in the oxidized core of PS 1.

The EPR signal of the $P700^+$-A_1^- radical pair was observed by time resolved EPR technique [41,42]. Snyder et al. [43] were the first who attributed A_1^- to vitamin K_1 on the basis of EPR measurements. Evans et al. [44] found the presence of a bound electron transport component in spinach chloroplasts showing EPR spectrum characteristic for ferredoxins in PS 1 (g = 1.95, g = 1.93 and g = 1.87 at 77 K). Later, Sonoike et al. [45] identified EPR signals from ferredoxins in PS 1: g = 1.94 for F_A, g = 1.92 for F_B, g = 1.89 for F_A /F_B mixture and g = 1.78 for F_X. These EPR spectra were recorded at 20 and 8 K. The EPR signal from F_X with g = 1.77 was observed in PS 1 particles at 10 K [46].

Moreover, EPR signals from other components of photosynthetic apparatus, namely the large signal in the g = 2.00 region from the chlorophyll free radical [35,44,47], EPR signal of oxidized carotene (Car^+) with g = 2.0033 and line width 1.1 mT and EPR signal of protochlorophyllide [48] were registered as well.

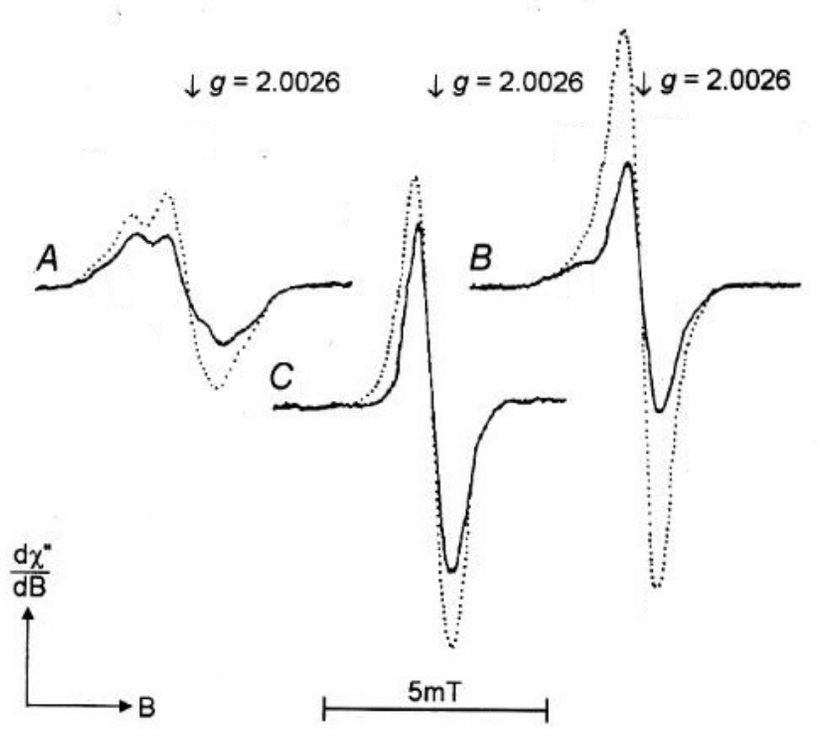

Figure 3. EPR spectra of spinach chloroplasts: control sample (A) and chloroplasts treated with 8 mmol dm^{-3} (B) or 40 mmol dm^{-3} (C) of $HgCl_2$. The full lines were recorded in the dark and the dotted ones in the light. Source: [26].

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4. Investigation of photosynthesizing organisms exposed to toxic metal stress

Several transient metals belong to very effective inhibitors of photosynthesis due to their ability to interact with amino acids occurring in proteins of photosynthetic apparatus or to release manganese ions from the water splitting complex. These processes can also be examined by EPR spectroscopy.

4.1. Copper

Copper is essential bioelement which occurs directly in photosynthetic electron transport chain, namely in the plastocyanin on the donor side of PS 1 [49] and in the light harvesting complex of PS 2 [50]. However, higher Cu concentrations result in the inhibition of photosynthesis due to interaction of Cu^{2+} with several parts of photosynthetic apparatus [51]. These interactions can be observable by EPR spectroscopy. It was found that Cu^{2+} ions at concentrations ~ 10 mmol dm^{-3} decrease the EPR signal intensity of $Tyr_Z^{\bullet}$ in spinach chloroplasts treated with Cu^{2+} ions [52-54]. The disappearance of both signals belonging to $Tyr_Z^{\bullet}$ and $Tyr_D^{\bullet}$ (shown in Fig. 3B and 3C for $HgCl_2$-treated chloroplasts [26]) was observed at higher Cu^{2+} concentrations (~ 50 mmol dm^{-3}) as well [54-57]. Moreover, incubation of chloroplasts with Cu^{2+} resulted in loss of the normal EPR signal from Q_A^- which is coupled to the non-heme Fe^{2+} on the acceptor side of PS 2 (the Q_A^--Fe^{2+} EPR signal). In the presence of excess Cu reduction of Q_A results in the formation of a free radical spectrum which is 0.95 mT wide and centred at g = 2.0044. This signal is attributed to $Q_A^{-\bullet}$ which is magnetically decoupled from the non-heme iron. This suggests that Cu^{2+} displaces the Fe^{2+} or severely alters its binding properties [53,58]. Moreover, application of higher Cu^{2+} concentrations resulted in displacement of Mn^{2+} ions from the manganese cluster and their release into interior of thylakoid membranes. This was documented by the appearing of six lines of hyperfine structure in EPR spectra of Cu^{2+}-treated spinach chloroplasts [54-57]. Similar effect for $HgCl_2$-treated chloroplasts is shown in Fig. 2B.

The Cu^{2+} ions appear to be predominantly associated with PS 2 proteins. In Cu-treated chloroplasts the formation of Cu(II)-protein complexes was confirmed by changes in EPR spectra of the applied Cu(II) compounds [55,57,59]. Using EPR spectroscopy it was found that Cu^{2+} is bound on two different sites of PS 2: one of them is situated near the Zn site that modulates electron transport between the quinones Q_A and Q_B and the second one occurs at the Fe site [60].

Interaction of copper with plastocyanin was presented by Bohner et al. [61] who found that in copper-treated *Scenedesmus* the content of this electron carrier dramatically varied with increasing external copper concentration.

4.2. Mercury

Mercury is a potential environmental contaminant which strongly inhibits photosynthetic processes in algae and higher plants [62]. Several sites of mercury action in both photosynthetic centres were determined. It was found that Hg^{2+} ions inhibit PET through PS 1 by interactions

with: i/ plastocyanin on the donor side of PS 1 [63-65]; ii/ ferredoxin [66,67]; iii/ F_B iron-sulphur cluster [68]. Hg^{2+} ions also damage PET through PS 2 [63,65,66] by interactions: i/ with OEC on the oxidizing side of PS 2 [26, 67, 69-72]; ii/ with the core of PS 2 (P680) [73]; iii/ with both quinone acceptors (Q_A and Q_B) on the reducing side of PS 2 [74,75]. Due to strong affinity of Hg^{2+} to CO, CN, CS and CSH groups the formation of organo-mercury compounds with amino acid residues in photosynthetic proteins was proposed as possible mechanism of the Hg^{2+} action [72,76,77].

Despite known Hg^{2+} action sites in the photosynthetic electron transport chain, EPR studies of mercury effect on photosynthetic apparatus were reported only by Sakurai et al. [78], Jung et al. [68], Šeršeň et al. [26] and Šeršeň and Kráľová [77]. Šeršeň`s group found that Hg^{2+} ions interact with the intermediates $Z^{\bullet}/D^{\bullet}$ what was reflected in reduction of both components of the EPR signal II (Fig. 3B and 3C). Moreover, EPR spectra of Mn^{2+} ions (Fig. 2B) which were released from manganese cluster into interior of thylakoid membranes confirmed interaction of Hg^{2+} with OEC [26]. A damage of the F_B iron-sulphur cluster in PS 1 after $HgCl_2$ treatment was demonstrated by EPR spectroscopy, while the EPR spectra of F_A and F_X remain unchanged [68,78]. The decay of EPR signal I after switching off the light in Hg-treated chloroplasts indicated the damage of direct cyclic and non-cyclic electron flow through PS 1 [77].

4.3. Cadmium

Cadmium belongs to the major heavy metal pollutants which have toxic effects on living organisms [79]. Cadmium exhibits several toxic effects on higher plants, which are caused by direct and indirect mechanisms of its action on plant photosynthetic apparatus [80]. The site of Cd action in photosynthetic apparatus was found to be situated on several sites of photosynthetic electron transfer chain within PS 2: i/ particularly in the OEC or in its vicinity on the donor side of PS 2 [67,77,81-84]; ii/ in the site of Q_A or Q_B on the acceptor side of PS 2 [69,85,86].

Some of the above mentioned sites of Cd^{2+} action were supported by EPR spectroscopy. It was found that Cd^{2+} decreased signal intensity of intermediates $Z^{\bullet}/D^{\bullet}$ and released Mn^{2+} ions from OEC into interior of thylakoid membranes [77]. These interactions of Cd^{2+} with OEC and $Z^{\bullet}/D^{\bullet}$ intermediates resulted in great increase of the signal I intensity [77]. Similarly to Hg-treated chloroplasts, application of Cd resulted in the damage of direct cyclic and non-cyclic electron flow through PS 1 what was demonstrated by kinetic behaviour of EPR signal I after switching off the light (Fig. 4) [77]. Addition of Cd^{2+} to Ca^{2+}-extracted PS 2 particles, which exhibited neither the multiline EPR signal nor g = 4.1 signal, did not restore these signals unlike Ca^{2+} addition when only the EPR g = 4.1 signal remained lost [85,87]. Ono and Inoue [88] found that Cd^{2+} substitution in Ca^{2+}-extracted PS 2 particles restored neither the multiline EPR nor g = 4.1 signals of S_2 state. A decrease in unstable free radical level in the leaves of wheat seedlings (*Triticum aestivum* L.) treated with low Cd concentrations (less than 3.3 mg kg^{-1} soil), followed by their significant enhancement with increasing Cd concentrations, were determined by EPR spectroscopy using spin trap [89].

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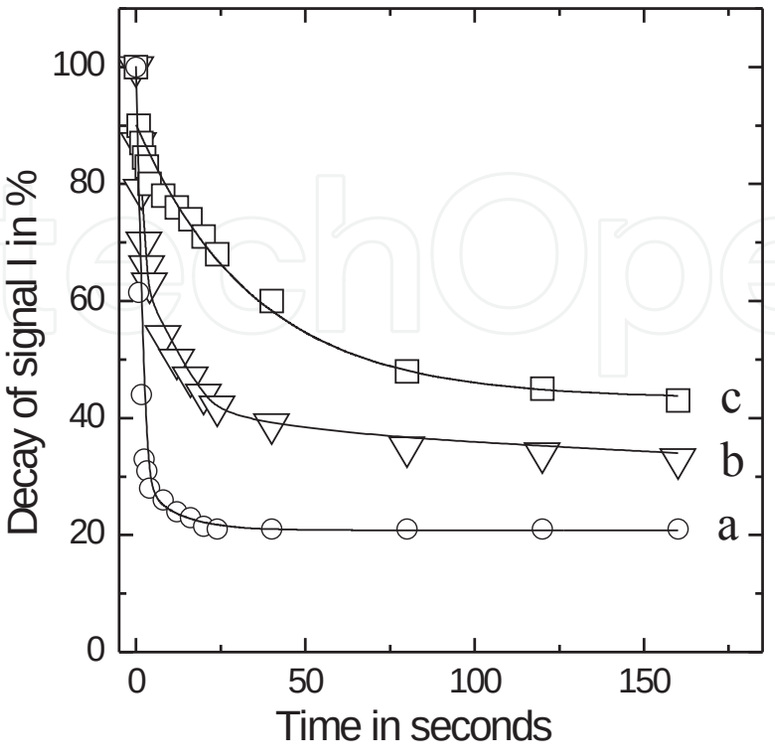

Figure 4. The kinetics of signal I of spinach chloroplasts after switch off the light in control chloroplasts treated with: 5 mmol dm^{-3} of DCMU (a), 0.05 mol dm^{-3} of $CdCl_2$ (b) and 0.05 mol dm^{-3} of $HgCl_2$ (c). Source: [77].

4.4. Zinc, chromium, selenium, nickel and iron

Karavaev et al. [90] investigated light-induced changes in the EPR signal I from oxidized reaction centres $P700^+$ of the photosynthetic apparatus of broad beans grown in aqueous solutions of zinc chloride. High concentrations of $ZnCl_2$ in the hydroponic medium slowed down the plant development and inhibited the light-induced production of oxygen and kinetics of redox transients of P700 induced by $ZnCl_2$ correlated with the changes in photosynthetic activity.

Frontasyeva et al. [91] studied the interaction of various chromium forms (Cr(III) and Cr(VI)) with *Spirulina platensis* biomass and found that from a nutrient medium the cells of this cyanobacterium mainly accumulated vitally essential form Cr(III) rather than toxic Cr(VI). Using EPR spectroscopy they demonstrated that the *Spirulina platensis* biomass enriched with Cr(III) was free from other toxic chromium forms. The toxicity of hexavalent chromium to photosynthesizing organisms is closely connected with generation of reactive oxygen species [92,93]. In the root tissues of some plants that were exposed to high concentrations of Cr(VI), the presence of intermediate Cr species, i.e., Cr(IV) or Cr(V) was confirmed by EPR spectroscopy studies by Micera and Dessi [94] as well as by low-frequency EPR experiments [95].

Labanowska et al. [96] used EPR spectroscopy to examine the alteration of radicals in wheat seedlings exposed for 2 days to selenium stress in two genotypes of Polish and one of Finnish wheat, differing in their tolerance to long-term stress treatment. The action of reactive oxygen species in short-term action of Se stress was confirmed by the reduction of PS 2 and PS 1 system activities. EPR studies showed changes in redox status (especially connected with Mn(II)/Mn(III), and semiquinone/quinone ratios) in wheat cell after Se treatment. Finnish wheat was recognized as the genotype more sensitive to short-term Se stress than the Polish varieties.

Using EPR spectroscopy it was found that Ni(II) complexes with *N*-donor ligands of the type NiX_2L_y (where X = Cl, Br, I, $ClCH_2COO$ or Cl_2CHCOO, L = nicotinamide or ronicol and y = 2 or 4) interact with Z^{+}/D^{+} intermediates, i.e. with tyrosine cation-radicals $Tyr_Z^{+\bullet}$, and $Tyr_D^{+\bullet}$ situated in D_1 and D_2 proteins on the donor side of photosystem 2 and with the manganese cluster in the oxygen evolving complex as well [97]. Similarly, the interaction with tyrosine radicals $Tyr_Z^{+\bullet}$ and $Tyr_D^{+\bullet}$ was also confirmed for Fe(III) complexes $[Fe(nia)_3Cl_3]$ and $[Fe(nia)_3(H_2O)_2](ClO_4)$, however release of Mn(II) from the oxygen evolving complex was observed only after treatment with $Fe(nia)_3(H_2O)_2](ClO_4)$ [98].

5. Investigation of photosynthesizing organisms exposed to herbicides

Herbicides are compounds used to kill weeds and unwanted plants. They also intervene in photosynthetic machinery and restrict photosynthetic electron transport due to their interaction with several components of photosynthetic apparatus. Therefore, EPR is very useful method for determination of the site of herbicide action.

Electron transfer from Q_A to Q_B is inhibited by a wide variety of plastoquinone (PQ) analogues that compete with PQ at the Q_B site. The most widely studied classes of inhibitors are the urea and triazine herbicides, such as 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and atrazine. Although their binding domains are likely to overlap with the urea/triazine herbicides on the Q_B site, "phenolic" inhibitors, which include bromoxynil, ioxynil, dinoseb and 2,4,6-trinitrophenol, appear to inhibit in a more complex fashion [99]. As mentioned above, different classes of herbicides apparently have different binding sites. DCMU-type herbicides bind to a 32 kDa polypeptide thought to be a regulatory protein associated with Q_A to Q_B electron transfer while phenolic herbicides bind to a 42 kDa polypeptide, which is probably a reaction centre protein. Herbicide-induced perturbations of these polypeptides might be expected to modify the EPR signals arising from reaction centre components. In particular, changes in the signal arising from Q_AFe might be expected since it arises from an interaction between two components, the semiquinone and the iron, which are located close to the herbicide binding sites [100].

Incubation of PS 2 membranes with herbicides results in changes in EPR signals arising from reaction centre components. Dinoseb, a phenolic herbicide which binds to the reaction centre polypeptide, changes the width and form of the EPR signal arising from photoreduced $Q_A^{-}Fe$ at g = 1.82. Orthophenanthroline slightly broadens the $Q_A^{-}Fe$ signal. These effects are attributed to changes in the interaction between the semiquinone and the iron. Herbicide effects can also

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be seen when Q_AFe is chemically reduced what is reflected by changes in splitting and amplitude of the split $Pheo^-$ signal. Dinoseb application also results in the loss of signal II_{slow}, in the conversion of reduced high-potential cytochrome b_{559} to its oxidized low-potential form and in the presence of transiently photooxidized carotenoid after a flash at 25 °C. These effects indicate that dinoseb may also deactivate OEC by accelerating the deactivation reactions of the water splitting enzyme [100].

Some commercial herbicides are known to bind to the site of the exchangeable quinone Q_B in the PS 2 reaction centre, thus blocking the electron transfer to Q_B what results in PET interruption. This effect can be demonstrated in EPR spectra of chloroplasts treated with DCMU recorded by continuous wave (cw) EPR apparatus at room temperature (Fig. 5). In Fig. 5 an increase of signal I intensity due to PET interruption from PS 2 is presented.

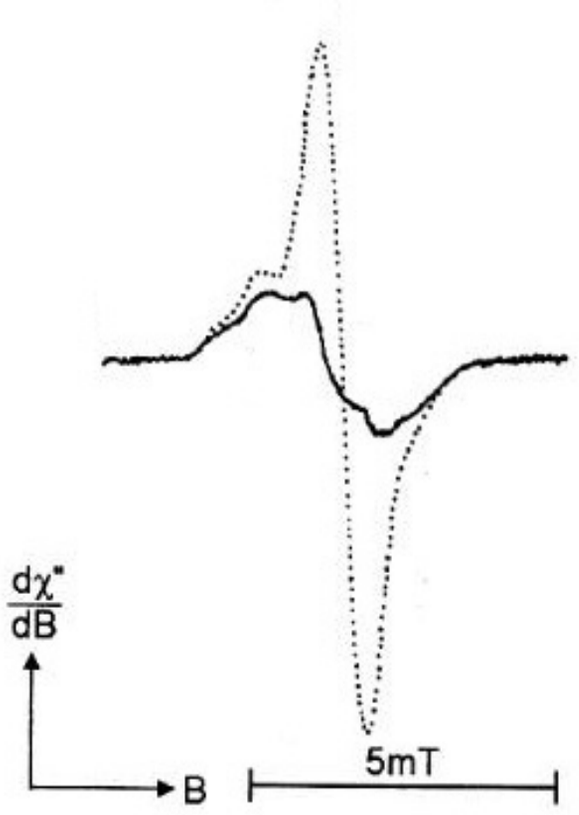

Figure 5. EPR spectrum of spinach chloroplasts treated with 100 μmol dm^{-3} of DCMU recorded in the dark (full line) and in the light (dotted line). Source: [24].

Some authors suggested that the herbicide-induced toxicity requires light and it is connected with chlorophyll-mediated $^1O_2$ generation (photooxidative stress). It is generally believed that as the energy is not being used for photosynthetic electron transfer, the chances of forming ^3Chl are increased, leading to $^1O_2$ formation and protein damage, similarly to the photodamage mechanism. This mechanism is also likely to apply to other herbicides. When electron transfer is blocked by herbicide binding, the level of the $S_2Q_A^{\bullet -}$ charge pair decays

by a charge recombination pathway that involves formation of a chlorophyll triplet in the heart of the reaction centre. This triplet is thus able to react with $^3O_2$ to give $^1O_2$ [101]. All above mentioned intermediates ($S_2Q_A^{\bullet-}$, ^{3}Chl and $^1O_2$) can be observed by EPR spectroscopy.

Tenuazonic acid is natural PS 2 inhibitor with several action sites [102,103]. Treatment of PS 2 particles with this bioherbicide resulted in generation of ROS such as $^1O_2$, $O_2^{-\bullet}$ and $^\bullet$OH which can be detected by EPR spectroscopy. Singlet oxygen was recorded as 2,2,6,6-tetramethylpiperidinoxyl radical (TEMPO) by EPR and production of $O_2^{-\bullet}$ and $^\bullet$OH was estimated by spin trap 5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide (DEPMPO) [104].

Photodamage of PS 2 in strong illumination of thylakoid membranes was documented by changes in the extent of the $Q_A^-Fe^{2+}$ and chlorophyll triplet EPR signals. In the presence of DCMU, a decrease of the $Q_A^-Fe^{2+}$ EPR signal (corresponding to the inhibition of oxygen evolution) and an increase of the chlorophyll triplet EPR signal indicated a possible overreduction of Q_A [105].

Multiphasic kinetic curve of light-induced EPR signal I from $P700^+$ was observed in the cyanobacterium *Synechocystis* sp PCC 6803 whereas in those treated with DCMU the PS 1 kinetics was monophasic [106].

Illumination of native NH_3-treated PS 2 membranes results in the appearance of an EPR signal at g = 2. It was suggested that this signal arises from perturbated S_3 state of manganese cluster [107]. González-Pérez et al. [108] observed the EPR signal with a g value of approximately 2.0043 and a line width of 0.1 mT which was induced under continuous illumination in the presence of peroxynitrite. In the absence of magnetic interaction with the non-heme Fe^{2+} this new EPR signal corresponds with the semireduced plastoquinone Q_A and it can be concluded that peroxynitrite impairs PS 2 electron transport in the Q_AFe^{2+} niche.

EPR spectroscopy was also used to determine the site of inhibitory action in the photosynthetic electron transport chain of many aromatic, heteroaromatic and amphiphilic compounds (in detail see in [109,110]).

6. Monitoring of the effect of gaseous environmental pollutants on photosynthetic apparatus

6.1. Ozone

Ozone is now regarded as the most important phytotoxic air pollutant, with long-term background concentrations increasing progressively. It is well known to have a negative impact on the photosynthetic apparatus of plant leaves and is thought to act via the formation of other ROS forms. Mehlhorn et al. [111] directly detected elevated levels of free radicals in plants exposed to ozone by EPR spectroscopy. It is generally thought that free radical generation in ozone stressed plants is enhanced by the direct reaction of ozone with biomolecules. This mechanism was supported by EPR measurements with freeze-dried samples of leaves

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from ozone-exposed wheat plants, showing a positive relationship between the free radical signal and the stress history of the plant [112].

In a C_3 grass, *Poa pratensis* L. and a C_4 grass, *Setaria viridis* Beauv., during exposure to O_3 both signal I (from $P700^+$ in PS 1) and signal II (from Tyr_D in the D_2 protein of PS 2) were stimulated by O_3. However, the fact that signal I observed in white light rose to the level of signal I in far-red light indicated reduced electron flow through PS 1 [113]. Reichenauer and Goodmann [114] found that after exposure to ozone in EPR spectra of freeze-dried samples from the same batches of plants an unidentified stable free radical appeared. The intensity of this radical signal increased with the duration of ozone exposure in leaves that received an additional ozone treatment.

Direct observations of radical signals obtained by EPR spectroscopy of intact, attached leaves of bluegrass *(Poa pratensis* L.) and ryegrass *(Lolium perenne* L.) and leaf pieces of radish *(Raphanus sativus* L.) during exposure to O_3 in air flowing through the spectrometer cavity have revealed the appearance of a signal with the characteristics of $O_2^{-\bullet}$. In each species, the signal only appeared after about 1 h of exposure to O_3, and then increased steadily over the next 4 h [115]. After prolonged exposure to ozone a free-radical signal with parameters similar to the superoxide anion free-radical signal was also formed in plant leaves of Kentucky bluegrass [116].

6.2. SO_2

The oxygen evolving enzyme of chloroplasts responsible for reduction of the PET carriers has been shown to be sensitive to SO_2 absorption by plants [117]. In SO_2-fumigated spinach leaves the time course of EPR signal I indicated that reduction of P700 by white light illumination was inhibited but dark reduction of P700 was not significantly affected. Photosynthetic O_2 evolution was also inhibited by SO_2 fumigation but all of these effects were reversible after removal of SO_2 [118].

Radish, Kentucky bluegrass and perennial ryegrass leaves subjected to high levels of sulphur dioxide (10–500 ppm) revealed the formation of signal I upon irradiation with broad-band white or 650 nm light, thereby indicating an interruption of normal electron flow from PS 2 to PS 1. Damage to the oxygen evolving complex and reaction centre of PS 2 was also detected through changes in signal II and Mn^{2+} signal [116]. These changes in the normal EPR signals were dose-dependent. Leaves subjected to low levels of sulphur dioxide (600–2000 ppb) revealed the disappearance of signal I after 3 hours of fumigation and the formation of a new free-radical signal with parameters similar to the sulphur trioxide free-radical signal. After prolonged exposure to sulphur dioxide a free-radical signal with parameters similar to the superoxide anion free-radical signal was formed in plant leaves [116].

7. Study of the thylakoid membrane arrangement under abiotic stress

The EPR spin label technique provides useful information about polarity of the local micro-environment near the spin label and the dynamic properties of the labelled site, which reflect

its conformational state. As spin probes are usually used the stable nitroxide radicals which are built into thylakoid membranes. From the line widths and line shapes of the EPR spectra of such built-in spin probe, various features of the local molecular environment and phase-transitions may further be deduced.

Using spin-labelled phosphatidylglycerol incorporated into thylakoid suspension it was found that Cu, Pb and Zn increased the surfaces available for lipid-protein interaction by dissociating membrane protein complexes [119]. EPR spectra of the lipid vesicles spin probed with n-doxylstearic acid (n-DSA) were used to explore the lipid rotational freedom at different depth of the bilayer. The EPR measurements indicated that the copper stress resulted in more tightly packed bilayers of the photosynthetic membranes with reduced acyl chain motion. Moreover, investigations of nitroxide radicals probing the bilayer at different depth suggested that changes in mobility associated with stress involve different parts of the bilayer in a similar way [120].

Line shape analysis of EPR spectra recorded as a function of temperature on concentrated suspensions of thylakoids with labelled 5-doxylstearic acid (5-DSA) allowed getting information about the fluidity of differently treated membranes. The immobilization of the spin probes in the hydrophobic part of the membranes was supported by experiments of Calucci et al. [121]. Another example of the use of spin labels 16-DSA (16-doxylstearic acid) or CAT-16 (*N*-hexadecyl-*N*-tempoyl-*N*,*N*-dimethyl-ammonium bromide) was carried out by Šeršeň et al. [3] who determined the inhibitory mechanism of *N*-alkyl-*N*,*N*-dimethyl amine oxides (ADAO) action upon PET. Above mentioned authors found that interactions of ADAO with thylakoid lipids of chloroplasts resulted in the decrease of the ordering and the microviscosity of lipid phase. The motion of a spin label after its incorporation into membranes will be limited and consequently changes in its EPR spectra occur. From these changes in EPR spectrum order parameter S characterizing the arrangement of the membrane can be calculated (in detail see in [3]). Application of membrane-active compounds results in perturbation in membrane structure. Consequently, the value of S parameter will be affected depending on the extent of membrane damage. From the dependences of the order parameter of thylakoid membranes S (determined from EPR spectra of CAT 16) on the concentration of ADAO for hexadecyl-, dodecyl- and hexyl-derivatives (Fig. 6A) and on the alkyl chain length of ADAO at the constant ADAO concentration (Fig. 6B) it is evident that the membrane perturbation increases with increasing concentration of membrane active compounds and that the most effective compound was dodecyl derivative.

Addition of membrane-active compound to chloroplasts containing spin labels results in an increase in the rate of molecular reorientation of spin label. Therefore, changes in the rotational correlation time τ_c (which is linearly proportional to the microviscosity of the environment in which the spin label is located) due to addition of membrane-active compound can also be used to characterize alterations in membrane arrangement (Fig. 7A, 7B).

It was shown that ADAO decreased the microviscosity of thylakoid membranes and the course of τ_c of 16 DSA spin label located in the thylakoid membrane on the alkyl chain

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length of ADAO at constant compound concentration 50 μmol dm^{-3} (Fig. 7B) was similar to that obtained for order parameter S (Fig. 6B), i.e. the lowest τ_c exhibited dodecyl derivate.

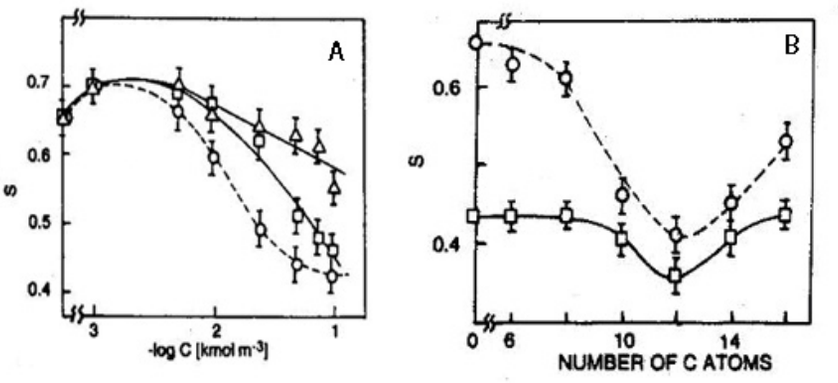

Figure 6. Dependences of the order parameter of thylakoid membranes S (determined from EPR spectra of CAT 16) on the concentration of ADAO for hexadecyl- (□), dodecyl- (o) and hexyl- (Δ) derivatives (A) and on the alkyl chain length of ADAO at the constant concentration of ADAO 50 μmol dm^{-3} (B); S was evaluated from EPR spectra of CAT 16 (o) and 16 DSA (□) spin labeles. Source: [3].

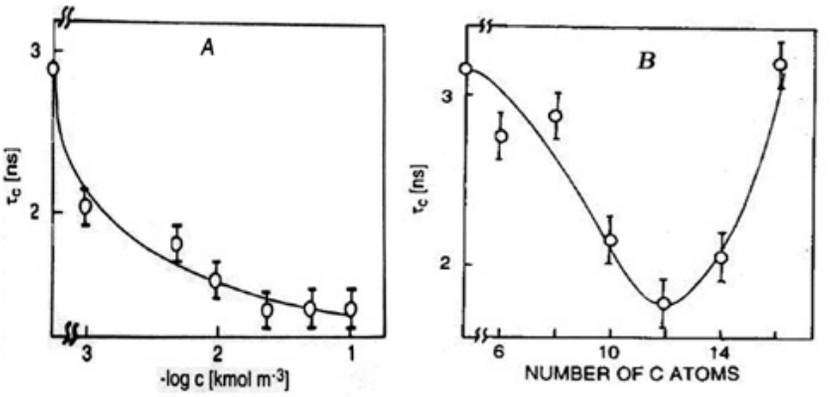

Figure 7. Dependences of rotational time τ_c of 16 DSA spin label located in the thylakoid membranes on the concentration of *N*-dodecyl-*N,N*-dimethylamine oxide (A) and on the alkyl chain length of ADAO at the constant concentration of ADAO 50 μmol dm^{-3} (B). Source: [3].

Alteration of membrane structure can also be caused by ionic amphiphilic compounds, including alkyl substituted quaternary ammonium salts [122-124]. Using EPR spectroscopy and spin label CAT 16 an increase of order parameter S was observed after application of low concentrations of 1-dodecyl-1-ethylpiperidinium bromide (DEPBr), followed with its decrease at higher DEPBr concentrations (Fig. 8A) [125]. These findings were in agreement with the results of oxygen evolution rate (OER) measurements which confirmed that OER was stimulated by low DEPBr concentrations (Fig, 8A). Because in the OER and in EPR experiments different chlorophyll content in chloroplast suspensions was applied, DEPBr concentration

within chloroplast organelles was calculated using DEPBr partition coefficient between chloroplast organelles and aqueous environment. From Fig. 8B in which the dependences of OER and order parameter S on the DEPBr concentration in chloroplast organelles are presented it is evident that OER stimulation and increase of order parameter S occurred in the same concentration range. Consequently, it could be assumed that OER stimulation is caused by changes in the arrangement of thylakoid membranes.

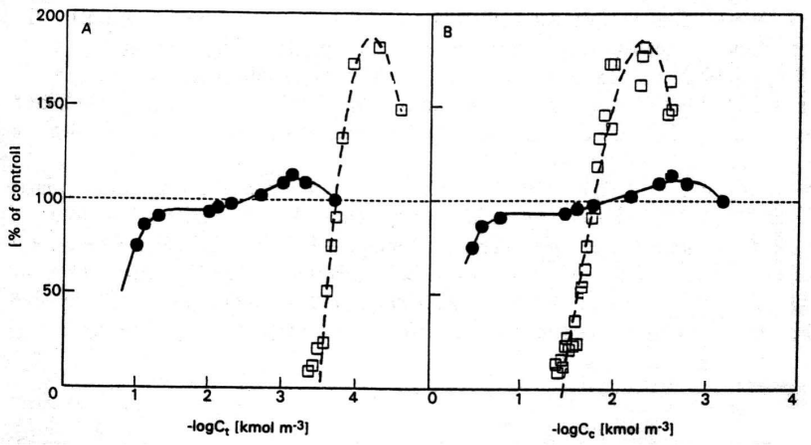

Figure 8. The dependence of the 2,6-dichlorophenolindophenol reduction (□) and the order parameter S (•) expressed as % of control sample upon concentration of 1-dodecyl-1-ethylpiperidinium bromide (DEPBr) in chloroplast suspension (A) and in chloroplast organelles (B). Source: [121].

Quartacci et al. [126] studied the effect of copper on the fluidity of PS 2 membranes by EPR measurements, using spin-probed fatty acids as probes. They found that due to treatment of PS 2 membranes (spin probed by means of 5- and 16-doxylstearic acids) with 50 μmol dm^{-3} Cu only the fluidity of the surface region of the bilayer close to the polar head group was reduced, while the fluidity of the inner membrane region of the bilayer did not show any change.

8. Study of photoinhibition in photosynthesizing organisms

When organisms that perform oxygenic photosynthesis are exposed to strong visible or UV light, inactivation of photosynthetic apparatus occurs. Under high light intensities the components of PET chain get damaged in a process called photoinhibition. It has been shown that PS 2 is the most susceptible pigment protein complex to photoinhibition. However, such organisms are able rapidly to repair the photoinactivated PS 2. Photoinhibition can be invoked by impairment of PS 2 acceptor side electron transport, and by the damage of PS 2 donor side [127]. Acceptor side induced photoinhibition takes place when reoxidation of quinones at acceptor side of PS 2 reaction centres is limited, e.g. when the plastoquinone pool is highly reduced. Under these conditions a charge recombination reaction between $P680^+$ and Q_A^- can take place, leading to a re-population of the primary charge pair $P680^+Pheo^-$. Recombination of this charge pair leads to the formation of the excited state of P680, both in its singlet and

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triplet state. ^{3}P680 reacts with O_2 leading to the formation of the highly oxidizing species $^1O_2$ [128]. In isolated thylakoids methylviologen (paraquat) was used to $O_2^{-\bullet}$ generation in the light in PS 1. $O_2^{-\bullet}$ was trapped to the spin trap by 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO) and this spin adduct was recorded by EPR spectrometer [128].

High-intensity illumination of thylakoids results in the impairment of PS 2 electron transport, followed by the degradation of the D_1 reaction centre protein. This impairment is caused by reactive oxygen species (ROS), which are formed in photosynthetic apparatus by high-intensity illumination. They are mainly supreroxide anion radical ($O_2^{-\bullet}$) and singlet oxygen ($^1O_2$). The formation of both above-mentioned ROS was confirmed by EPR spectroscopy. $^1O_2$ is generated by interaction of molecular oxygen with excited triplet of chlorophyll formed via charge recombination of radical pair ^{3}[P680$^+$ Pheo$^-$] [129]. Singlet oxygen was detected by following the formation of 2,2,6,6-tetramethylpiperidine-1-oxyl, a stable nitroxide radical yielded in the reaction of singlet oxygen with the sterically hindered amine 2,2,6,6-tetramethylpiperidine. Singlet oxygen, a non-radical form of active oxygen, was detectable only in samples undergoing acceptor-side-induced photodamage [130]. During both types of photoinhibition also other free radicals were detected as spin adducts of the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide, and identified on the basis of hyperfine splitting constants of the EPR spectra.: i/ The acceptor-side induced process was accompanied by the oxygen dependent production of carbon centred (alkyl or hydroxyalkyl) radicals, probably from the reaction of singlet oxygen with histidine residues. (ii) Donor-side induced photoinhibition was dominated by hydroxyl radicals, which were produced in anaerobic samples, too. The production rate of these radicals, as well as D_1 protein degradation, was dependent on the possibility of electron donation from manganese ions to PS 2. The marked distinction between the active oxygen forms produced in acceptor- and donor-side induced photoinhibition are in agreement with earlier reports on the different mechanism of these processes [130]. The two types of trapped ROS radicals are presented in Fig. 9.

Ogami et al. [131] observed that after 4 hours cultivation of cyanobacterium *Thermosynechococcus elongatus* cells under high light conditions, the S_2 multiline signal was undetectable. Ivanov et al. [132] confirmed the impairment of PS 1 by measuring of EPR signal intensity of oxidized P700 under high light stress.

The UV-A (320-400 nm) component of sunlight is a significant damaging factor of plant photosynthesis, which targets the PS 2 complex. UV-A irradiation results in the rapid inhibition of oxygen evolution accompanied by the loss of the multiline EPR signal from the S_2 state of the water-oxidizing complex. Gradual decrease of EPR signals arising from the $Q_A^-Fe^{2+}$ acceptor complex, Tyr_D, and the ferricyanide-induced oxidation of the non-heme Fe^{2+} to Fe^{3+} was also observed, but at a significantly slower rate than the inhibition of oxygen evolution and the reduction of the multiline signal. The amplitude of signal II_{fast}, arising from Tyr_Z in the absence of fast electron donation from the Mn cluster, was gradually increased during UV-A treatment. However, the amount of functional Tyr_Z decreased to a similar extent as Tyr_D as shown by the loss of amplitude of signal II_{fast} that could be measured in the UV-A-treated particles after Tris washing. It was concluded that the primary damage site of UV-A irradiation is the catalytic manganese cluster of the water-oxidizing complex, where electron transfer to

Tyr_Z and $P680^+$ becomes inhibited. This damaging mechanism is very similar to that induced by the shorter wavelength UV-B (280-320 nm) radiation, but different from that induced by the longer wavelength photosynthetically active light (400-700 nm) [7].

Strong UV-A light from a laser inactivated the oxygen-evolving machinery and the photochemical reaction centre of PS 2. The release of Mn^{2+} ions from PS 2 during incubation of thylakoid membranes in very strong UV-A light was documented by recording of EPR spectra of Mn^{2+} ions in thylakoid membranes from *Synechocystis* cells [9].

Pospisil et al. [134] indicated that $^{\bullet}OH$ is produced on the electron acceptor side of PS 2 by two different routes: i) $O_2^{-\bullet}$, which is generated by oxygen reduction on the acceptor side of PS 2, interacts with a PS 2 metal centre, probably the non-heme iron, to form an iron-peroxide species that is further reduced to $^{\bullet}OH$ by an electron from PS 2, presumably via $Q_A^{-\bullet}$; ii) $O_2^{-\bullet}$ dismutates to form free H_2O_2 that is then reduced to $^{\bullet}OH$ via the Fenton reaction in the presence of metal ions, the most likely being Mn^{2+} and Fe^{2+} released from photodamaged PS 2. H_2O_2 causes extraction of manganese from OEC, inhibits its activity and photosynthetic electron transfer, and leads to the destruction of the photosynthetic apparatus. EPR spectroscopy documented an increase in the level of P700 photooxidation, an decrease of the rate of its subsequent reduction in the dark and an increase of free Mn^{2+} ions after addition of H_2O_2 [135].

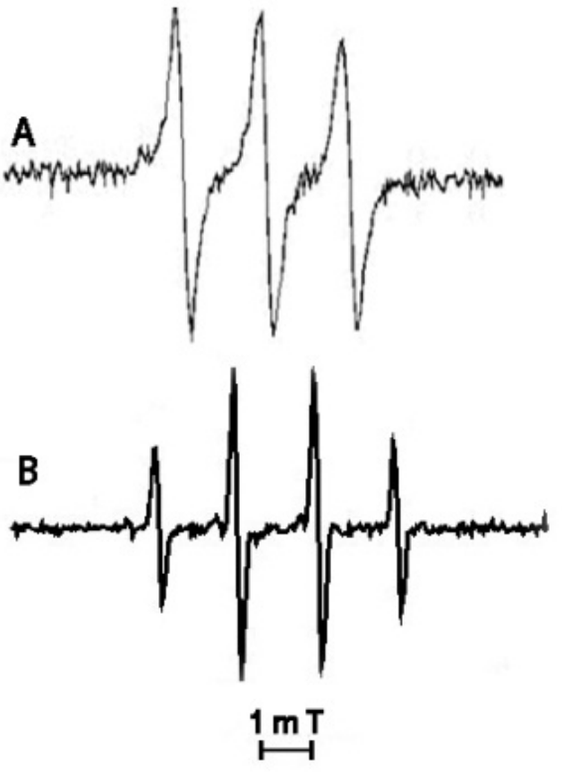

Figure 9. EPR detection of singlet oxygen trapped by TEMP (2,2,6,6-tetramethylpiperidine) (A), and hydroxyl radicals trapped by DMPO (5,5-dimethyl-pyrroline *N*-oxide) (B) in thylakoid membranes exposed to the light. Source: [133].

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The other concept of the monitoring of detrimental action of strong light or other unfavourable living conditions is inspection of the typical hyperfine structure of monodehydroascorbate (MDA). Oxygen is a natural electron acceptor in the PET chain, during which the superoxide anion radical ($O_2^{-\bullet}$) is formed in the thylakoids. $O_2^{-\bullet}$ is reduced to H_2O_2 by CuZn superoxid dismutase (SOD). During the reduction of H_2O_2 by ascorbate peroxidase, MDA is formed, which is detectable by EPR in photoactive conditions. Plants, which are exposed to an excess of radiation, cannot completely utilize it in photosynthesis. This excess radiation can exhibit a damaging effect on photosynthetic apparatus. Leaves are equipped with several protective mechanisms involved in preventing oxidative and photoinhibitory damage. The resulting negative effects on plants depend on the capacity of cellular systems to scavenge ROS and to prevent or to repair harmful effects of light on the PET components. MDA is a long lasting anion radical, it can be detected by EPR spectroscopy at room temperature and serves as an endogenous probe for oxidative stress. Under optimal conditions the concentration of MDA in the leaves is too low to be detected in the light or darkness. Under environmental stress when the rate of oxygen activated species surpasses the MDA reducing capacity, MDA can be detected by EPR, as was the case in leaves treated with paraquat or aminotriazole (Fig. 10). In illuminated leaves paraquat is photoreduced to the paraquat radical that rapidly reacts with O_2 to $O_2^{-\bullet}$, $O_2^{-\bullet}$ is disprotonated to H_2O_2 by SOD at the site of its production, leading to an increase of H_2O_2 level in the chloroplasts. Scavenging of H_2O_2 gives rise to MDA signal that is light dependent [136]. Impact of air pollutants, chemicals, herbicides, photooxidants and unfavourable environmental conditions like drought, high temperatures and even mechanically induced injuries lead to increase of the MDA concentration which can be monitored by EPR spectroscopy. Thus, by simple measurements a proof of oxidative stress can easily be performed. Therefore, the MDA EPR signals can be used as a general marker of stress situations [137]. Light-induced MDA radical production was not detectable by EPR spectroscopy in untreated broad bean leaves, but it was observed after exposing the leaves to UV-B irradiation. After this pretreatment, a low level of MDA radicals was also detectable without illumination [138].

However, we would like to note that beside of investigations focused on the effects of strong visible light and UV irradiation on photosynthesizing organisms, effects of low light on plants were investigated as well. In these experiments mainly chlorophyll fluorescence characteristics were estimated [139,140], however there are some papers in which the use of EPR technique is reported. Paddock et al. [141] investigated reaction centres from *Rhodobacter sphaeroides* by EPR at high and low light intensities. They found that decay kinetics of EPR signal after switch off light exhibited two phases. The fast decay with a time constant τ = 30 ms belongs to the decay of $D^{+\bullet}Q_A^{-\bullet} \rightarrow DQ_A$, where D is the reaction centre in *Rhodobacter sphaeroides*. Slow phase had a longer time constant τ = 6 s. However, when the sample was illuminated at lower light intensity, the relative amplitude of the slow phase was larger indicating that the slow descending component is connected with the decay of the $D^{+\bullet}Q_B^{-\bullet}$ state. EPR technique was also used to study light-induced alteration of low-temperature interprotein electron transfer between PS 1 and flavodoxin [142] utilizing the fact that deuteration of flavodoxin enables the signals of the reduced flavin acceptor and oxidized primary donor, P_{700}^+, to be well-resolved at X- and D-band EPR. While in dark-adapted samples photoinitiated interprotein electron transfer does not occur at 5 K, for samples prepared in dim light significant interprotein

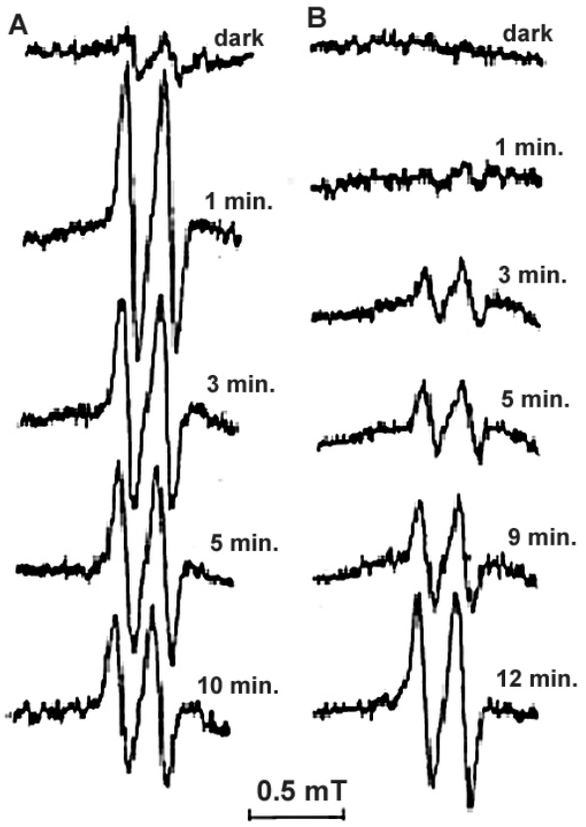

Figure 10. EPR spectra of MDA in *Vicia faba* leaves treated with paraquat (A) or aminotriazole (B). The irradiance was 1000 W m^{-2}. Numbers in A and B mark the time in light when EPR spectra were recorded. Source: [136].

electron transfer occurred at this temperature and a concomitant loss of the spin-correlated radical pair $P_{700}{}^{+}A_{1A}{}^{-}$ signal was observed. This indicated a light-induced reorientation of flavodoxin in the PS 1 docking site that allows high quantum yield efficiency for the interprotein electron transfer reaction.

9. Study of photosynthesizing organisms exposed to drought and chilling stress

9.1. Drought

Water deficits cause a reduction in the rate of photosynthesis. Limitation of carbon dioxide fixation results in exposure of chloroplasts to excess excitation energy. When carbon dioxide fixation is limited by water deficit, the rate of active oxygen formation increases in chloroplasts as excess excitation energy, not dissipated by the photoprotective mechanisms, is used to form superoxide and singlet oxygen which can be detected by EPR spectroscopy. Superoxide formation leads to changes suggestive of oxidative damage including lipid peroxidation and a decrease in ascorbate level [143].

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Elevated levels of free radicals were detected in leaves of drought-stressed barley plants during and after release of drought stress compared with those seen in the controls. However, they returned rapidly to the control levels after release of the stress. On the other hand, a sizeable increase in the level of a mononuclear Fe(III) complex was seen in the droughted samples (compared with the levels in the watered controls), and these elevated levels remained after release of the stress [144].

EPR quantification of superoxide radicals revealed that drought acclimation treatment led to 2-fold increase in superoxide radical accumulation in leaf and roots of wheat (*Triticum aestivum*) cv. C306 with no apparent membrane damage. However, under subsequent severe water stress condition, the leaf and roots of non-acclimated plants accumulated significantly higher amount of superoxide radicals and showed higher membrane damage than that of acclimated plants indicating that acclimation-induced restriction of superoxide radical accumulation is one of the cellular processes that confers enhanced water stress tolerance to the acclimated wheat seedlings [145].

When germinating *Zea mays* L. seeds were rapidly desiccated, free radical-mediated lipid peroxidation and phospholipid deesterification was accompanied by a desiccation-induced generation of a stable free radical associated with rapid loss of desiccation tolerance, which was detected by EPR spectroscopy. At the subcellular level, the radical was associated with the hydrophilic fraction resulting from lipid extraction. Modulation of respiration using a range of inhibitors resulted in broadly similar modulation of the build-up of the stable free radical [146].

EPR measurements showed that also microsomes isolated from wheat leaves exposed to drought, and from leaves exposed to drought followed by watering, generated significantly higher amount of hydroxyl radical as compared to microsomes isolated from control leaves, suggesting higher production of $^{\bullet}OH$ in the cellular water-soluble phase after drought and watering, as compared to control values. Lipid radicals combined with the spin trap α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone (4-POBN) resulted in adducts that gave a characteristic EPR spectrum with hyperfine coupling constants of a^N = 1.58 mT and a^H = 0.26 mT but no significant effect on lipid radical content was measured after drought and drought followed by watering, as compared to controls [147].

The values of signal II for drought-stressed *Sorghum bicolor* and *Pennisetum glaucum* plants were found to be lower by 3 to 9%, similar to non-drought-stressed plants after light stress. However, after a combination of light and drought stress, signal II was decreased by 11 to 32%, indicating that the donor side of PS 2 is also affected by drought stress and high irradiance [148].

9.2. Chilling

At chilling temperature and low light intensity PS 1 is selectively inhibited while PS 2 remains practically unchanged [149]. The activity of PS 1 in cucumber leaves was selectively inhibited by weak illumination at chilling temperatures with almost no loss of P700 content and PS 2 activity. The sites of inactivation in the reducing side of PS 1 were determined by EPR and flash photolysis. EPR measurements showed the destruction of iron-sulphur centres, F_x, F_A

and F_B, in parallel with the loss of quantum yield of electron transfer from diaminodurene to $NADP^+$ [45].

EPR spectra of stearic acid spin labels incorporated into spinach thylakoids can be used to monitor membrane changes during freezing and changes in the EPR parameters can be directly correlated to the extent of functional freeze damage. Jensen et al. [4] used stearic acid spin labels to study the effect of freeze damage to thylakoid membranes microviscosity. They determined changes in EPR parameters either as a function of temperature or during freezing at −15 °C as a function of time and found that an empirical parameter h_+/h_0 (ratio of height of a low field line component h_+ over height of the central line h_0) proved to be very sensitive to minute changes in membrane structure. The observed changes in line shapes were interpreted as an increase in mobility and/or orientation of the lipids following the swelling of thylakoids, however, they did not indicate a disorganization of the lipid phase. Freeze-induced changes in the EPR parameters were found to be strongly dependent on the osmotic conditions of the incubation medium and they were similar to changes observed by transferring thylakoids from an isotonic to a hypotonic medium, i.e., by swelling osmotically flattened thylakoids [150].

Broadening of the EPR signals of 16-doxyl stearic acid in chloroplast membranes of frost-sensitive needles of *Pinus sylvestris* L. and changes in the amplitudes of the peaks were observed upon a decrease in temperature from +30 °C to −10 °C, indicating a drastic loss in rotational mobility. The EPR spectrum of thylakoids from frost-tolerant needles at −10 °C was typical of a spin label in highly fluid surroundings. However, an additional peak in the low-field range appeared in the subzero temperature range for the chloroplast membranes of frost-sensitive needles, which represents spin-label molecules in a motionally restricted surrounding. The domains with restricted mobility could be attributed to protein-lipid interactions in the membranes [151].

EPR study of phospholipid multibilayers, obtained from two cultivars of thermally acclimated wheat of different frost revealed two breaks in the motion of the spin-labelled fatty acid 2-(14-carboxyte-tradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl, for both cultivars (+3 °C, –17 °C and +5 °C, –18 °C, respectively) when grown at 22 °C. Exposure of the resistant cultivar to cold (+2 °C) resulted in the shift of the onset of the apparent phase-separation temperature from +3 °C to –16 °C. However, the sensitive cultivar was unable to do so [152].

Intact tissues and microsome preparations from root tips of coffee seedlings subjected for 6 days to temperatures of 10, 15, 20 and 25 °C in darkness were investigated by EPR using fatty acid spin probes 5-, 12- and 16-doxylstearic acid. It was found that at the depth of the 5th and 16th carbon atom of the alkyl chains the nitroxide radical detected more rigid membranes in seedlings subjected to 10 °C compared with 15 and 25 °C. Membrane rigidity induced by chilling was interpreted as due to lipid peroxidation that could have been facilitated by higher density of peroxidizable chains below the membrane phase transition. At the C-12 position of the chains the probe showed very restricted motion and was insensitive to chilling induced membrane alterations [153].

Incubation at 5 °C and a moderate photon flux density (PFD) decreased the rate of $O_2^{-\bullet}$ production by 40% and 15% in thylakoids from *Spinacia oleracea* L. and 20 °C grown *Nerium*

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oleander L. (chilling-insensitive plants), but increased the rate by 56% and 5% in thylakoids from *Cucumis sativus* L. and 45 °C grown *N. oleander* (chilling-sensitive plants). The rate of $O_2^{-\bullet}$ production increased in thylakoids when the rate of electron transfer to NADP was reduced what could explain differences in the susceptibility of thylakoids from chilling-sensitive and chilling-insensitive plants to chilling at a moderate PFD, and is consistent with the proposal that $O_2^{-\bullet}$ production is involved in the injury leading to the inhibition of photosynthesis induced under these conditions [154].

10. Other potential uses of EPR in photosynthesis

10.1. Oxymetry

Oxygen concentration in thylakoids can be monitored by EPR using some spin probes (nitroxide probes, phtalocyanine, etc.). Determination of oxygen concentration is based on physical phenomenon of an oxygen-induced line broadening in the EPR spectrum of selected stable free radicals due to their collisions with molecular oxygen. EPR oxymetry offers high sensitivity (typically 10^{-12} mol dm^{-3} at 100 μmol dm^{-3} nitroxide during 1 s), small sample volume and permits monitoring of time-resolved changes in oxygen concentration on millisecond time-scale [155-157].

10.2. Measurements of pH in thylakoids

There are two most frequently used EPR methods for ΔpH measurements in chloroplasts. They are based on pH-indicating spin probes. The first method consists in the calculation of Δ pH from the partitioning of permeable amine spin probes (usually TEMPAMINE; 2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amine) between the thylakoid lumen and the suspending medium. By addition of a membrane-impermeable paramagnetic compound, chromium oxalate (which broadens the EPR signal from TEMPAMINE in the external medium), into chloroplast suspension, the probe molecules occurring outside and inside of the thylakoid can be visualized. The second method is based on the measurement of EPR spectra of pH-sensitive spin probes (imidazoline nitroxide radicals) loaded into the vesicles [158].

11. Conclusion

Electron paramagnetic resonance (EPR) spectroscopy is a useful method to study photosynthetic processes because it can monitor the presence of compounds with unpaired spins in the photosynthetic apparatus. Intermediate compounds with unpaired spins are formed directly in photosynthetic apparatus during photosynthetic electron transport in light reactions of photosynthesis and more than 20 different EPR signals covering all electron transfer components can be observed. Moreover, the formation of reactive oxygen species (ROS) during photosynthetic processes in plant chloroplasts can be recorded by EPR spectroscopy as well. This method enables to study the effects of various abiotic stresses (e.g. gaseous pollutants

such as ozone and SO_2, heavy metals, herbicides, heat, cold, salt, drought, excess of the light as well as UV-A and UV-B radiation and dim light) on photosynthetic apparatus. The formation of complexes between toxic metal ions and photosynthetic proteins as well as excessive formation of ROS caused by various abiotic stress factors can also be monitored by EPR spectroscopy. Moreover, EPR spin probe technique is suitable to study changes in the arrangement and viscosity of photosynthetic membranes in the presence of an abiotic stressor. EPR spectroscopy is a valuable tool to study photodynamic processes (short living radicals or radical pairs, as well as kinetics of their creation or decay) in photosynthesis. Beside this, EPR spin probe method can be used to determine the concentration of photosynthetically released oxygen (EPR oxymetry) or to measure ΔpH in chloroplasts.

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