Oxidative Stress of Crops Monitored by EPR

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Z. Naturforsch. **48c,** 766–772 (1993); received March 3/July 1, 1993

Herbicides, Paraquat, Amitrole, Ascorbic Acid Radicals

Treatment of leaves of spinach, corn, and peas with the herbicides paraquat, amitrole or acifluorfen leads to oxidative stress resulting in a light driven drastically increased production of ascorbic acid radical (monodehydroascorbic acid, MDAA) which could be demonstrated by *in vivo* EPR analysis. A discrimination of the MDAA formation between the action of electron uncouplers and catalase inhibitors can be achieved by observation of the radical rise kinetics. Significant MDAA signal intensities are detected in the darkness likewise. These signals are probably due to the action of ascorbic acid oxidase activated by membrane destruction.

Introduction

In previous papers [1, 2], we described EPR investigations of the needles *in vivo* to monitor the action of the anti-oxidative defense system of spruce trees (*Picea abies* [L]). The most significant signals in this context were those of the ascorbic acid radical (monodehydroascorbic acid, MDAA) which was detected under illumination after action of ozone or aminotriazole known for inhibition of catalase action, or paraquat which uncouples the electron transport in photosystem I under formation of reactive oxygen species. A comparison of the radical contents with the concentration of the diamagnetic precursor, ascorbic acid (AH) clearly shows the advantage of the EPR method. The MDAA radicals are produced in a very early state of oxidative stress whereas the concentration of AH changes only if the regeneration is not sufficient.

During these investigations we detected an obviously more sensitive reaction of deciduous plants and a significant difference in the rate of formation and decay of the MDAA depending on the nature of herbicides used to induce oxidative stress. For this reason, we decided to systematically investigate leaves of spinach, corn, and peas by EPR after treatment with several herbicides. Investigations of entire photosynthetic organs by EPR spectroscopy is an exception in studies on environmental stress by reactive oxygen species [3] or intense illumination [4]. EPR measurements with leaves or leaf fragments provide the following important advantages as compared to the generally used chloroplast or membrane preparations for investigations of paramagnetic compounds. There is: no time consuming sample preparation, no artifacts introduced by preceding sample preparation, easy variation in the composition of the atmosphere, and maintenance of the fully functioning state in the cavity which may last up to several days under special conditions [3].

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Verlag der Zeitschrift für Naturforschung, D-72072 Tübingen
0939–5075/93/0900–0766 $ 01.30/0

Materials and Methods

Plant material

Spinach and corn were grown in pots in a home mix of natural soil with compost under field conditions near Tübingen, and peas in pots in a commercially available soil. The pots were located at a window at the East front of our institute.

Herbicide treatment

The approximately four weeks old plants were treated with 1% aqueous solution of paraquat or 3-amino-1,2,4-triazole or a 1% suspension of acifluorfen using a spray device. After a defined incubation time the leaves were harvested and immediately subjected to radical determination by EPR spectroscopy for a period of several days.

EPR measurements

From spinach or corn plants leaf sections of approximately 1 × 2 cm were cut off and placed with their lower surface on a quartz plate with the help of Pelican glue which is EPR inactive in darkness as well as under illumination. The size of the pea leaves fits well with dimensions of the cavity, therefore no sectioning was necessary. The measurements were done at room temperature using a Bruker X-band spectrometer, by accumulating 50 scans using a FF-lock under the following conditions: micro wave energy, 5 mW, 12.5 kHz field modulation, amplitude 400 mG, range 50 G, scan time 41 s, gain 1.0×10^5, time constant 82 ms.

The radical intensities were obtained by dividing the average of the peak to peak amplitudes of both MDAA lines (s. Fig. 1) by that of the photosignal I, in order to get results independent of the loaded cavity Q value [5]. The reproducibility of the values obtained mainly depends on the signal-to-noise ratio. Typical values are 100:3. The absolute concentrations of the MDAA radicals observed are approximately 10^{-7} mol^{-1}. These values strongly depend on the experimental conditions (s. Fig. 2).

The data characterized "illuminated" are obtained from light-dark difference spectra, because only this difference provides informations concerning light driven reactions. The time depending measurements were usually done by locating the magnetic field at the peak of the low field MDAA EPR line. Modulation amplitude, time constant and conversion time were adjusted to the problem under investigation predominantly in order to optimize the signal-to-noise ratio and avoid obscuring of the phenomenon under study.

The exchange of the atmosphere surrounding the sample was done by flooding the cavity with the appropriate gas.

Results

Treatment of the leaves with one of the herbicides paraquat, amitrole or acifluorfen leads to remarkable ascorbic acid radical concentrations within a few hours. In Fig. 1 typical spectra are shown. The two sharp lines attributed to the MDAA superimpose in darkness the photosignal II next to the center and under illumination photosignal I on its low field side.

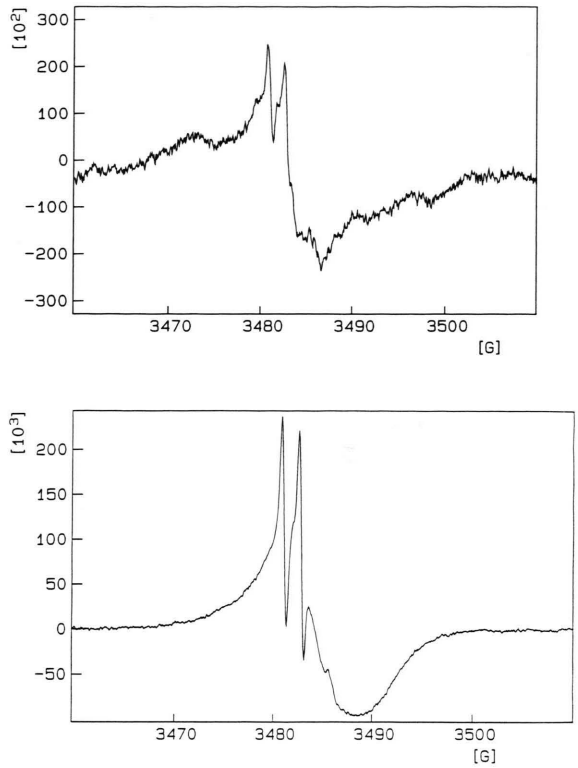

Fig. 1. EPR spectra of a corn leaf 4 h after treatment with paraquat. Above: dark signal; below: light-dark signal.

Investigation of the MDAA signal intensity dependence on the time after treatment reveals a very fast change of radical concentration as shown in Fig. 2 for spinach, tobacco and corn. The time required for maximum radical concentration was only several hours whereas in the case of spruce needles [2] the signal intensity maximum was observed after a few days.

The decrease of the EPR signal intensity below the limit of detection coincides with the death of the plant in all cases.

In contrast to these results, a single treatment of the plants investigated with amitrole or acifluorfen – not shown – does not ruin them immediately. However, remarkable MDAA signals under illumination are observed, likewise. The occurrence of the maximum radical concentration is delayed in comparison to the paraquat treatment. All samples reveal ascorbic acid radicals in the dark adapted leaves, too. The concentration is approximately

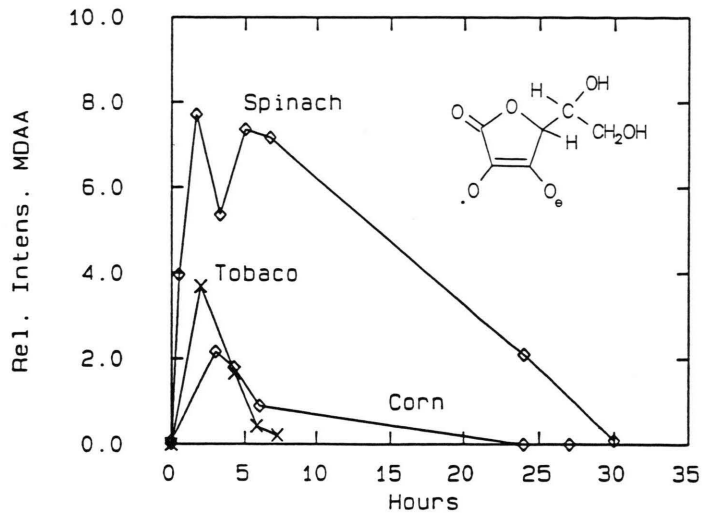

Fig. 2. Time dependence of light driven MDAA signal intensities *versus* time after treatment with paraquat.

ten-fold higher as compared to the untreated control plants. The data obtained are compiled in Table I.

After treatment with the herbicides several samples reveal some maxima of the radical concentration with different peak heights under illumination as well as in darkness, for example (s. Fig. 5). In order to avoid confusion, the time needed to produce the first maximum of the MDAA signal-amplitude is given in Table I. Furthermore, additional signals are observed at higher field with respect to MDAA lines, particularly after treatment with paraquat.

In all cases ambient oxygen plays a key role in the MDAA formation. Consequently, the EPR signal drastically depends on the surrounding atmosphere. This is shown by a typical experiment in Fig. 3.

The experiment starts as in Fig. 3 above. The leaf is in the cavity in the darkness under air, after 10 s the light was switched on. A small but significant increase of the signal intensity is observed immediately. This is due to photosignal I which superimposes the MDAA spectrum. The latter is built up slowly reaching a steady state concentration after 75 s. At 100 s the atmosphere was changed to nitrogen resulting in a decrease of the signal to the starting level. The experiment continues in the lower part of Fig. 3, with replacement of the nitrogen by air. This leads to the regeneration of the MDAA radicals. Their concentrations rise at first and subsequently decrease if the light is switched

Table I. Impact of herbicides monitored by MDAA.

Plant	Herbicide	Darkness		Illumination		Additional signals
		1. Maximum	rel. intensity	1. Maximum	rel. intensity	
Spinach	acifluorfen	2 h	0.2	7 h	1.0	
	paraquat	2 h	0.15	2 h	8.0	2.0026 2.0031
	amitrole	13 h	0.31	4 h	5.6	
Corn	paraquat	3 h	0.26	3 h	2.2	2.0030 2.0037 2.0027
	amitrole	18 h	0.15	20 h	2.7	
Peas	paraquat	4 h	0.2	4 h	1.7	2.0030
	amitrole	4 d	0.37	4 d	21	
Picea abies [L.]	paraquat*	6 d	0.13	6 d	5.0	
	amitrole*	9 d	0.05	9 d	1.9	

* Under field conditions.

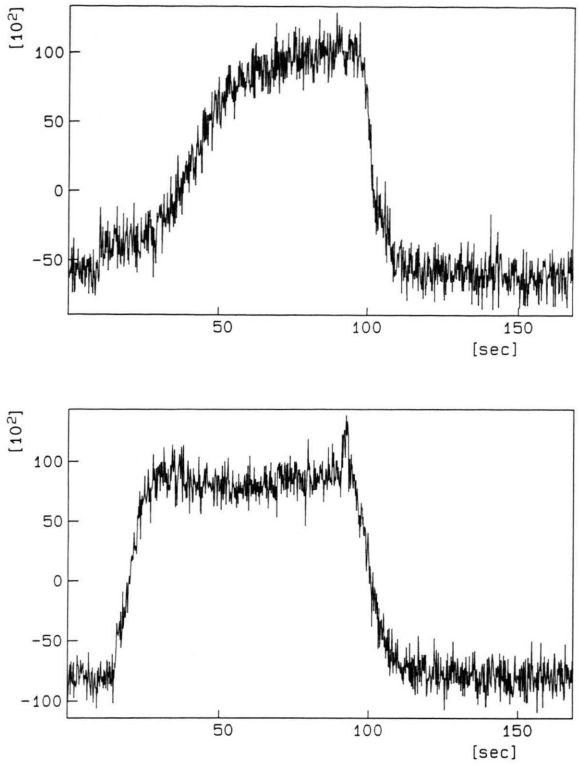

Fig. 3. Intensity of the MDAA signal of a spinach leaf after treatment with amitrole under variable conditions of illumination and atmosphere.

off after 90 s. Amitrole and acifluorfen treated leaves of other plants reveal qualitatively analoguous pictures and, therefore, confirm the importance of reactive oxygen species in the mechanisms responsible for the herbicide action. Interesting is a comparison of the light driven reactions under normal atmosphere, that means in the experiment described above the first increase and last decrease in Fig. 3 with the experiment after treatment with paraquat, given in Fig. 4.

This experiment starts in air in the dark. Immediately after illumination a very steep increase and a maximum of the radical concentration is observed. 15 s later the MDAA signal shows a time independent intensity. The light was switched off after 60 s, and the signal intensity decreased, subsequently. Because the time scale in Fig. 3 and 4 is the same, a simple comparison reveals a much faster MDAA generation and a significantly slower decay observed with paraquat treated spinach leaves.

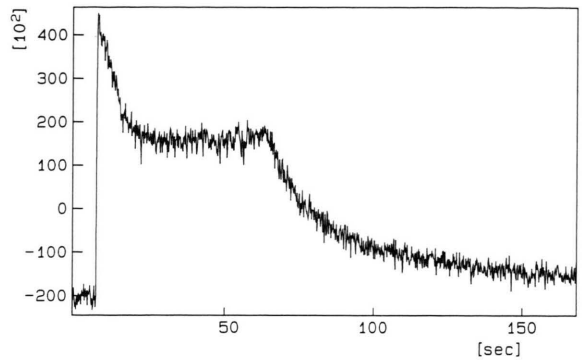

Fig. 4. Intensity of the MDAA signal of a spinach leaf after treatment with paraquat under variable illumination.

The investigation of ascorbic acid radical concentration over a period of several hours shows significant variations of the signal intensity as well in darkness as under illumination (s. Fig. 5, for example). This behavior was found in all plants analyzed and was independent of the herbicide used. Therefore, we assume that this phenomenon is due to defense reactions of the plant and not a consequence of imperfect distribution of the drug in the plant, which particularly for paraquat is very unlikely, according to the fast translocation of this herbicide.

Significant MDAA signals are not only detected under illumination but also in darkness if the leaves are treated with the herbicide under discussion. However, the intensities observed under illumination are essential larger. In Fig. 5, both the

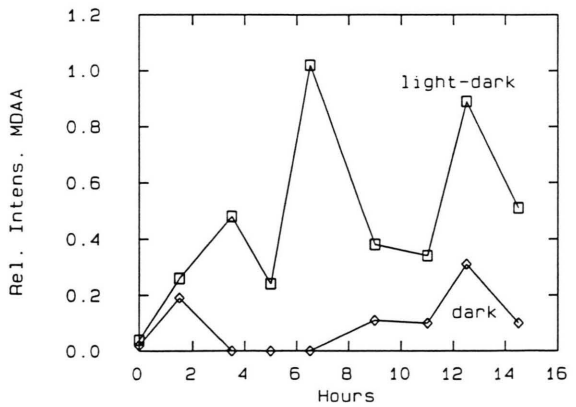

Fig. 5. MDAA signal intensity *versus* time of acifluorfen treated spinach leaves.

MDAA signal intensities for the two experiments *versus* the incubation time are given.

Along with MDAA there are other radicals detected in the darkness as well as under illumination, as already mentioned. In Fig. 1, for example, the shoulder at the high field side of the photosignal I is due to other paramagnetic species produced in the course of oxidative stress. Their g-factors are smaller compared to the MDAA and the lines are sharp ($\Delta H = 0.5$ G) (s. Table I and Fig. 6). Therefore, no substantial hyperfine interaction is observed.

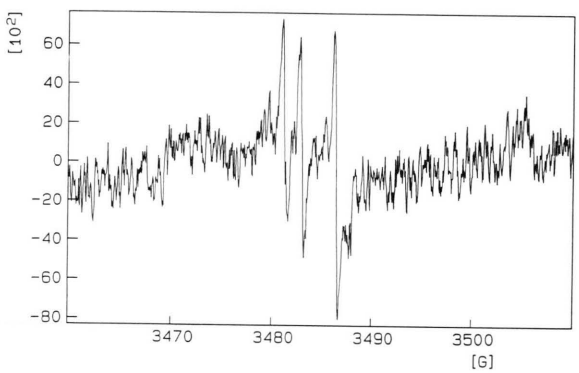

Fig. 6. Dark EPR spectrum of a spinach leaf 400 min after treatment with paraquat.

Discussion

For the production of the ascorbic acid radicals we assume, according to the literature, mainly the enzymatic oxidation of ascorbic acid by H_2O_2. The origin of the peroxide is due to either detoxification of the superoxide radical formed by interaction of paraquat [6] with the electron transport chain, or inhibition of catalase by amitrole which acts in the peroxisome [7, 8] to avoid high peroxide concentrations in this compartment. The literature discussing mechanisms responsible for the action of acifluorfen on green leaves is very controversial [8, 9]. However, the impact of the three herbicides on the plant photosynthetic organs can easily be monitored by EPR spectroscopy.

The concentration of the MDAA radical strongly depends on the illumination at the one hand, and the plant investigated on the other, as well as on the herbicide used. The quantitative determination of the MDAA concentration is very time consuming and was done only for several exemplary samples. For the investigation of the time dependence (s. Fig. 2) and for the plant sensitivity (s. Table I) relative concentrations are sufficient. Using these values, the data collected in Table I show at first that herbicides penetrate leaves of deciduous plants much more easily than those of conifers. The production of MDAA is faster after paraquat treatment in all cases, and the radical intensities are significantly higher under illumination compared to the signals observed in darkness. This indicates a different mechanism for the light independent radical generation which will be discussed below. Furthermore, the ascorbic acid in deciduous leaves is completely consumed after approximately one day, whereas the spruce needles resist the paraquat treatment for at least 12 days.

The experiments in different atmospheres shown for example in Fig. 3, indicate the key role of oxygen for the formation of MDAA as well in the light driven processes as in darkness. The rate of the radical decay (Fig. 3 above) and radical formation (Fig. 3 below) is identical indicating that only diffusion processes are responsible for the kinetics observed. In contrast to this the light dependent reactions, signal increase (Fig. 3 above) and decay (Fig. 3 below) are completely different according to several reactions. The distinct maximum of the radical concentration observed after the light was switched off (s. Fig. 3 below) requires a light driven reduction pathway for MDAA which contributes to the steady state concentration. Probably the electrons of the photosystem I are directly used in a non-enzymatic reaction.

In all cases investigated (s. Table I) significant light driven MDAA signals are observed, indicating the formation of reactive oxygen species independent of the herbicide used. The different physiological mechanisms responsible for the MDAA generation may be distinguished by the observation of their rise and decay kinetics. Comparison of Fig. 3 and 4 indicates a much slower light driven radical formation after treatment with amitrole compared to paraquat. In this case the rate constants for the two preceding electron transfer reactions are known [10]. The reduction of paraquat proceeds with $k = 8.4 \times 10^{10}\ \mathrm{M^{-1}\ s^{-1}}$, and the reaction of the paraquat radical with oxygen is also very fast ($k = 7.7 \times 10^{8}\ \mathrm{M^{-1}\ s^{-1}}$). The values were determined by puls radiolysis in aqueous solution.

As a result of this, the overall reaction is so fast that no discrimination between the MDAA signal and the photosignal II can be detected under the conditions applied, whereas the formation of both in the spectrum (s. Fig. 1) superimposed signals can be time resolved (s. Fig. 3 above). The radical decay reaction rates are likewise different in both cases. Thus, by registration of the rise and decay kinetics an unambiguous decision between herbicide action with the electron transport chain on the one hand and the catalase on the other can be achieved. Further work to study the kinetics more quantitatively by computer simulation are in progress.

The observation of remarkable MDAA concentrations in darkness (s. Fig. 5 and 6) is at first glance surprising, because of the well known instability of this radical. Therefore, the signals observed have to be interpreted in terms of a steady state situation. A simple oxidation of ascorbic acid by air oxygen cannot account for the radical formation because EPR experiments *in vitro* reveal a very low rate for this reaction. Therefore, the oxidation of ascorbic acid in darkness should be a catalyzed process. The catalyst could be the enzyme ascorbate oxidase which is liberated by membrane destruction. In order to confirm this assumption we destructed the cell membranes of spruce needles by insertion into liquid nitrogen. By EPR investigations of these needles strong MDAA signals are detected. The intensity decreases with a first order kinetics $k = -4.75 \times 10^{-3}\ s^{-1}$, the correlation coefficient is 0.9770 (s. Fig. 7).

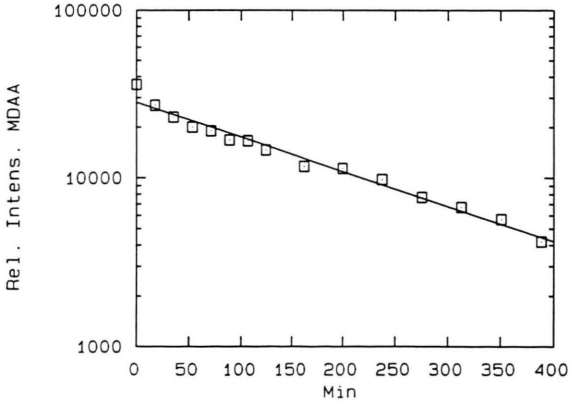

Fig. 7. Decrease of the MDAA in spruce needles after insertion in liquid nitrogen *versus* time, ordinate logarithmic scale.

Contamination of the needles with heavy metals particularly Fe^{3+} ions was excluded by this procedure, and the metal containing enzymes are stable under the conditions applied. The enzyme ascorbate oxidase detected by Szent-Györgi [11] is located in the membranes. The chemical properties of this enzyme have been reviewed [12], but its function is still under discussion. Perhaps, the concentration of molecular oxygen in the tissue is controlled in this way.

EPR signals of comparable intensities could be observed by investigation of zucchini peels with the EPR spectrometer (not shown). This sample shows in darkness the expected photosignal II, superimposed with the HFS of the MDAA present in low concentration. After addition of the substrate ascorbate the radical concentration increases drastically.

The protective function of ascorbic acid is, furthermore, demonstrated by the detection of additional radicals in the darkness as well as under illumination (s. Fig. 6). The g-factors observed strictly exclude paramagnetic oxygen species and favour carbon centered radicals, instead. However, any addition of radicals to lipid or carotenoid moieties leads to paramagnetic species for which at least a hyperfine structure, due to β-coupling, is expected. For this reason an unambiguous assignment of these signals cannot be done at the time being. However, the EPR spectra indicate a destruction of the photosynthetic organs. This interpretation is consistent with the fact that signals of this type are predominantly observed several hours after treatment for example with paraquat, at this time merely low MDAA concentrations are observed indicating an almost complete consumption of the ascorbic acid pool.

Acknowledgements

This work was supported by the Fonds der Chemischen Industrie and the Deutsche Forschungsgemeinschaft (DFG).

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