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THE OZONE EFFECTS ON THE CHLOROPLASTS EXTRACTED FROM *RUMEX PATIENTIA* – AN EPR INVESTIGATION

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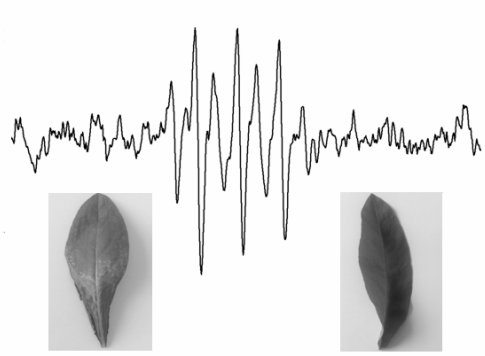
The aim of this work was to evidence the effects of ozone exposure on the oxidative process in chloroplasts isolated from *Rumex patientia* plants. The visible alteration of plants exposed to ozone was related to the reduced antioxidative capacity of the chloroplasts components.
Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) evidenced that the stress proteins isolated from chloroplasts of *Rumex patientia* leaves exposed to the ozone action are more mobile than those extracted from unexposed leaves. This can be due either to conformational changes or to modification of proteins primary structure under the ozone action. The presence of the reactive oxygen species (ROS) generated by exposure of plants to ozone in chloroplasts have been evidenced by spin trapping method using 5,5-dimethyl-1-pyrroline N-oxide (DMPO). Furthermore the antioxidative capacity of the plant components has been analyzed following the changes in electron paramagnetic resonance (EPR) spectra of the TEMP/TEMPO mixture. The EPR experiments showed that antioxidative capacity of the chloroplast is significantly decreased in the case of exposed plants.



INTRODUCTION

Ozone represents one of the most common gaseous pollutants responsible for blocking the photosynthetic pathways in plants. The ozone molecules in high concentration cause the impairment of the photosynthetic electron transport chain through the formation of damaging free radicals.[1] Ozone can affect membrane permeability, enzyme activity and causes leaf chlorosis as well as necrosis. It enters into plant through stomata and subsequently reacts with cell wall and membrane components leading to the production of reactive oxygen species (ROS) thus damaging the cellular components.

The ozone facilitate the formation of ROS like hydroxyl radical (HO·) superoxide anion (O_2^{-}·) and singlet oxygen ($^1O_2$). In high concentration ROS are harmful to organisms and when these species exceed the defense mechanisms, the cells are under oxidative stress. This process involves lipid peroxidation, protein oxidation, damage of nucleic acid and enzyme inhibition. However, under normal conditions about 1% of O_2 consumed by plants is diverted to produce ROS in various subcellular loci such as chloroplasts, mitochondria and peroxisomes.[2]

The antioxidative systems of an organism involve both enzymatic and nonenzymatic antioxidants. Among the nonenzymatic antioxidants present in

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cells ascorbate, glutathione, carotenoids, and phenols are the well known.[3] The presence of short lived free radicals is responsible for plant senescence. Therefore the premature senescence is a symptom frequently associated with the exposure of plants to ozone.

Elucidation of different plant responses ranging from senescence to rapid necrosis can be generally explained by the relative dose of ozone that the plant receives. The balance of reactive oxygen species concentration versus antioxidant scavenging potential helps to decide the cell´s fate.[4]

When the hydroxyl radical is formed, being extremely reactive attacks numerous target molecules indiscriminately. There are no specific scavengers for this radical within plants.

In this study we aimed to show the negative impact of the ozone action on the *Rumex patientia* by observing visible alteration of the plants exposed and analyzing the presence of short lived radicals in the chloroplasts extracted from the exposed and unexposed plants using EPR spectroscopy, including spin trapping method.

RESULTS AND DISCUSSION

The ozone was discharged in the chamber atmosphere using an electrical generator. Plants were adapted to the laboratory condition for three days prior separation in two groups. One of them was then exposed to the ozone action for eight days. After this period the leaves of plants were examined. Fig.1 presents photos of leaves exposed and unexposed to the ozone action which evidence the lesions caused by ozone exposure. These consist in the presence of yellow spots on the leave surface, dehydration and tissue necrosis.

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has been performed in order to evidence the changes induced in the proteins isolated from the chloroplasts after the ozone treatments. SDS-PAGE is a method used for separating proteins according to their size. The anionic surfactant sodium dodecyl sulfate (SDS) binds to most proteins in a constant weight ratio. Proteins heated in presence of SDS denature into their primary polypeptides and gain an essentially identical negative charge density. These polypeptides, migrating in an electric field towards the positive anode, can be separated in a porous gel depending on their size with smaller proteins migrating faster than the larger ones.[5]

In Fig. 2 the SDS-PAGE of purified samples of *Rumex patientia* unexposed and exposed to ozone (for eight days) is shown. The approximate molecular weight of the proteins isolated from *Rumex patientia* chloroplasts was compared by a standard molecular ruler (Precision Plus Protein Standards from BIORAD). Standard proteins with molecular weights of 250, 150, 100, 50, 37, 25, 20, 15, 10 kDa were used as markers.

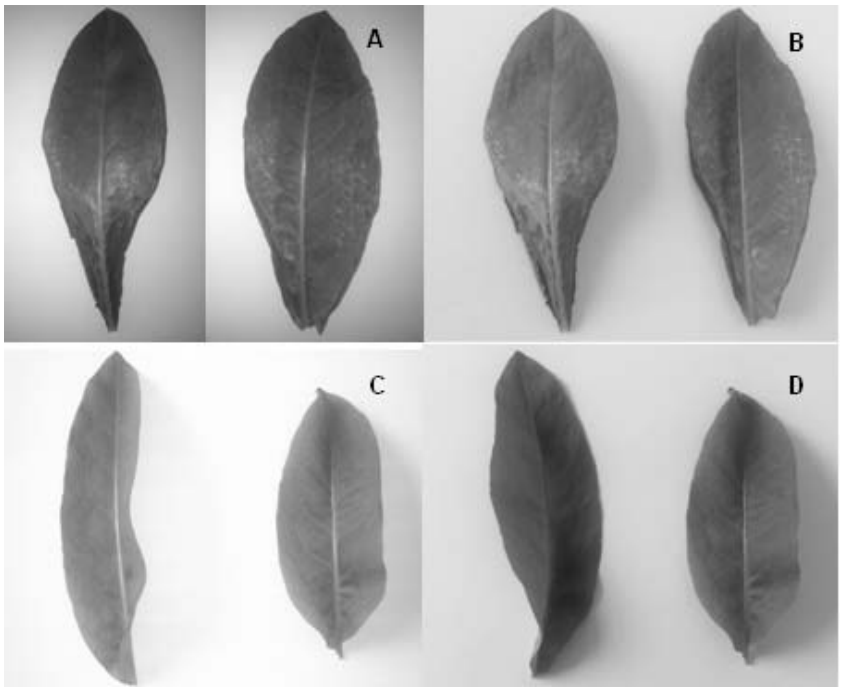

Fig. 1 – Images of exposed plants to ozone for 8 days (A and B) and unexposed plants to ozone (C and D).

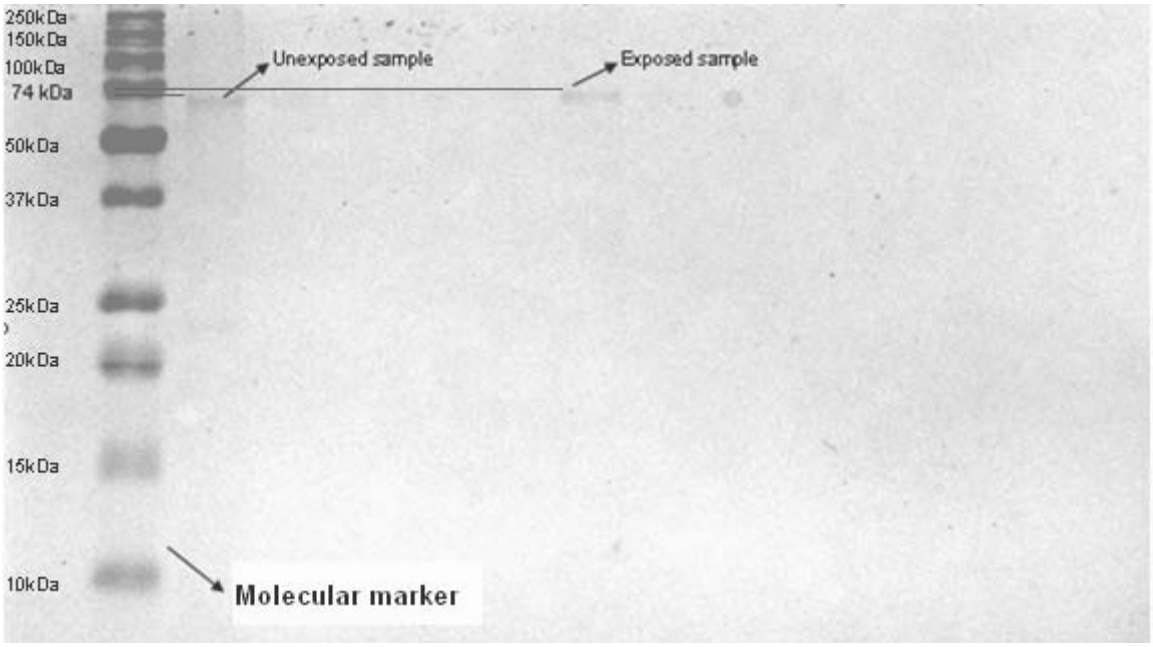

Fig. 2 – SDS-PAGE of the supernatant from the chlorophyll solution;
Staining Coomassie Brilliant Blue M_w marker [kDa] in the left lane. Concentration of the gel 12%.

The proteins with a molecular mass of approximately 74 kDa according to the SDS gel can be assigned to the "heat shock stress protein" family. Heat shock proteins (HSPs) are characterised by molecular weight within the range of 68 - 110 kDa and are expressed in response to environmental stresses.[6-8] These include thermal conditions (heat or cold), water availability, pollutants (heavy metals, ozone, acid rains), mineral and nutrient availability.[9]

The Coomassie staining solution (0.4g Coomasie Blue R-250 0.4%, 40 mL methanol 40%, 10 mL acetic acid 10% and distilled water to 100 mL) of the SDS-gel revealed that the protein band corresponding to the extract from the exposed plants to ozone is less intense than the band corresponding to the extract from the plant kept in normal atmosphere (unexposed), but is characterized by a higher mobility. This observation is an indicative of structural changes of this class of proteins which determine either conformational or primary structure changes. Similar effect was described in literature in the case of isolated light –harvesting proteins of photosystem II membranes upon visible irradiation.[10]

Exposure to the ozone can generate various ROS species in various locations like chloroplasts, mitochondria, plasma membrane, peroxisomes, apoplast, endoplasmatic reticulum, cell wall.[3] To identify the presence of the ROS in the chloroplasts extracts from *Rumex patientia* leaves exposed to ozone actions the DMPO and TEMP were used as spin traps (fig 3). DMPO is a spin trap often used to detect reactive species like HO·, or superoxide generated by exposure of plants to the ozone.

The EPR measurements on samples obtained by mixing the re-suspended pellets from plants exposed to ozone with fresh solution of DMPO (0.1M) prepared in buffer B3 did not evidence the formation of the spin adducts with ROS. This observation suggests that such reactive species already had reacted with chloroplasts components during separation procedure or if they are still present their concentration is bellow the detection limit of EPR method. In order to check if ROS can be generated in chloroplasts, ozone enriched air had been passed through a suspension of pellet in buffer B3, in the presence of DMPO and the EPR measurements evidenced the presence of DMPO adducts (Fig. 4 A-C). Similar experiment for buffer B3 in the presence of DMPO has been performed (Fig. 4D).

The EPR spectra A-C from Fig. 4 represent correspond to spectra of DMPOX spin adduct which often result by decomposition of DMPO spin adducts corresponding to HO·, $O_2^{\cdot -}$. In this situation we cannot exactly attribute the formation of this adducts to a certain type of ROS generated by ozone exposure. Even so, the formation of the oxygen singlet specie ($^1O_2$) during ozone exposure can be involved in various oxidative processes of proteins, fatty acids, DNA.[3]

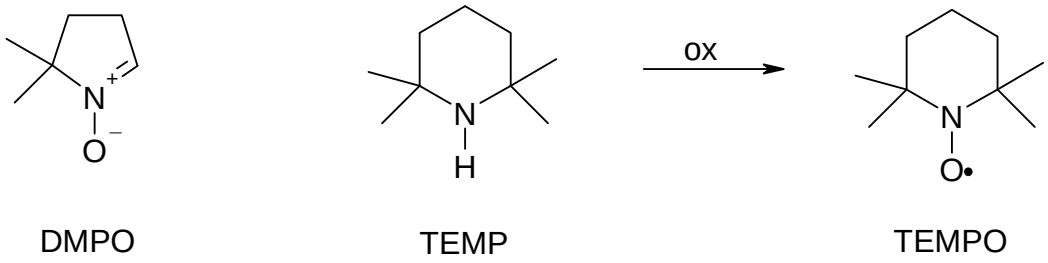

Fig. 3 – Chemical structures of spin traps used in this study and TEMPO.

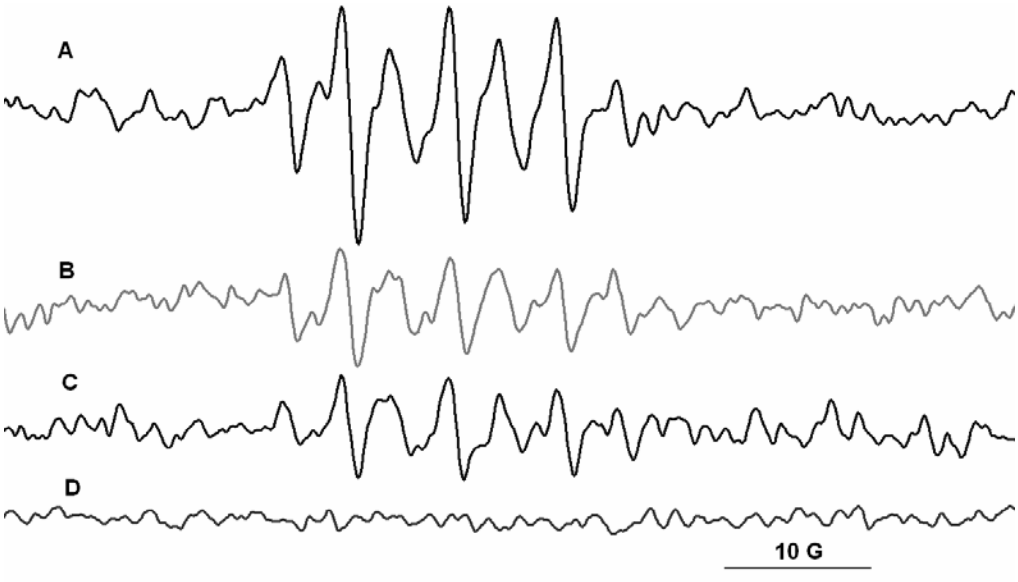

Fig. 4 – EPR spectra of exposed to ozone extract and DMPO: A – EPR spectra of DMPO in extract after exposure to O_3 initial; B – EPR spectra of DMPO in extract after exposure to O_3 after 2 minutes; C – EPR spectra of DMPO in extract after exposure to O_3 after 7 minutes; D – EPR spectra of DMPO in Buffer without chloroplast extract.

At the chloroplast level, the $O_2^{\cdot -}$ radical can be generated in condition of overloading of electron transfer chain in stress condition.[11] The formation of DMPOX spin adduct has been identified from the splitting constants: a_N = 7.29 G, a_H = 4.04 G obtained by spectral simulation using WINSIM program. These values are similar with those reported in literature.[12-13]

A specific spin trap for $^1O_2$ is TEMP (Fig. 3).[10-13] In the presence of $^1O_2$, TEMP is oxidized to TEMPO radical which compared with all ROS or their spin adducts is a stable nitroxid. Although the reactive used in this study were new, in case of TEMP it was observed that this reactive contains as impurity TEMPO radical. The a_N value of TEMPO dissolved in TEMP has the hyperfine splitting value a_N = 15.5 G. When TEMP is added to a buffer solution, TEMPO is distributed between organic phase represented by TEMP and aqueous phase (buffer). The hyperfine splitting (a_N) value for TEMPO in water was found 17.15 G. However we found that TEMPO is preferably localized in organic phase represented by TEMP. The presence of TEMPO as impurity in TEMP does not represent a disadvantage in our experiment, as the increased concentration of TEMPO radical in aqueous phase can prove the presence of oxygen singlet.

By adding TEMPO/TEMP mixture to the freshly prepared suspension containing chloroplast from plants exposed to the ozone show a typical three line spectrum for TEMPO (fig 5 A). The sample obtained by mixing the chloroplast extract with TEMP/TEMPO exhibits a stronger EPR signal compared with that corresponding to TEMPO/TEMP and is characterized by a_N value of 17 G. The TEMPO radical formed by oxidation of TEMP in the presence of chloroplasts is localized thus, in large proportions in aqueous environment. Recording subsequently the EPR spectra for 30 minutes it was observed the disappearance of the signal of TEMPO from aqueous environment. The EPR spectra remain almost unchanged in organic environment. This result can be rationalized as

follows. The presence of singlet oxygen is evidenced by increased signal of TEMPO in aqueous media which contains chloroplast components. In the next step, the natural scavengers which can be found in chloroplasts reduce the TEMPO to TEMPO-H, which is a diamagnetic molecule. Because this experiment refers to the extract obtained from plants exposed to ozone, it is likely that their defense system was partially annihilated. The TEMPO dissolved in TEMP (which remains unreacted) is not reduced.

The similar EPR experiment involving the chloroplast extract obtained from unexposed plants indicates that TEMPO is rapidly reduced to TEMPO-H. As it is evidenced in Fig. 6, the EPR signal almost disappeared after 8 minutes. In the same time, can be noticed the initial presence of TEMPO between two phases, and the fact that the TEMPO is reduced from both phases.

The reduction of TEMPO proves that the defense plant system is efficient and radical scavenger neutralizes the presence of radicals. As it was mentioned in the introduction, the antioxidative defense systems in plants are constituted both from nonenzymatic and enzymatic components. Ascorbic acid is one of the most representative nonenzymatic antioxidants. In the presence of radicals, ascorbic acid is oxidized in two steps to dehydroascorbic acid. The intermediate is the ascorbyl free radical (Fig. 7), characterized by a resonance structure which make possible to be detected in certain condition directly by EPR spectroscopy. The EPR spectrum of ascorbyl radical shows two lines.

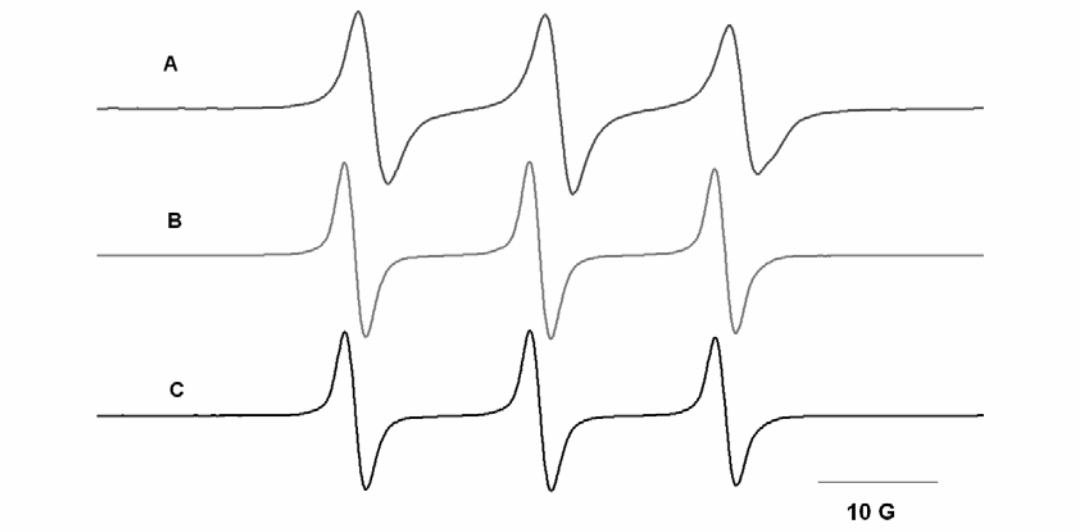

Fig. 5 – EPR spectra of exposed to ozone leaves extract and TEMP
A) immediately after adding TEMP, B) after 15 minutes, C) after 30 minute.

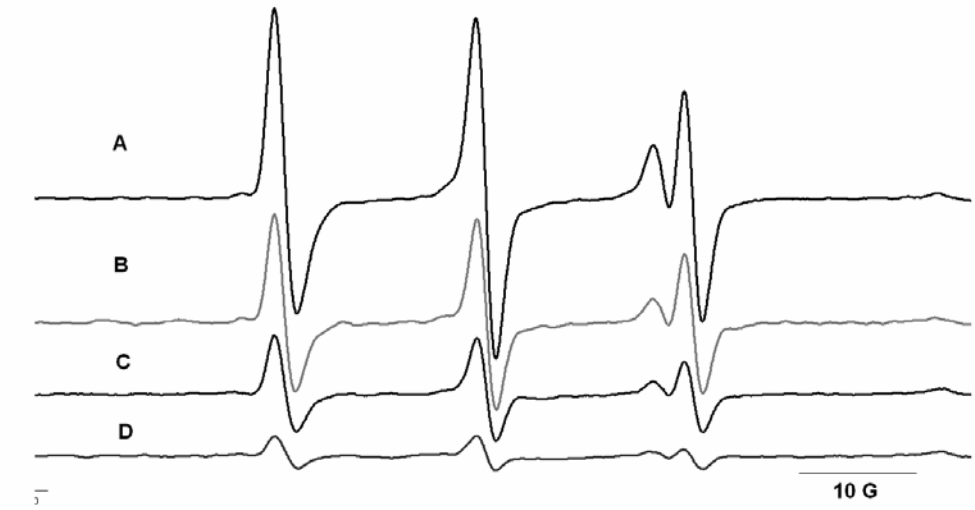

Fig. 6 – EPR spectra of TEMPO after mixing with unexposed plant extract
A) immediately after mixing TEMP with the extract, B) after 2 minutes, C) after 4 minutes, D) after 8 minutes.

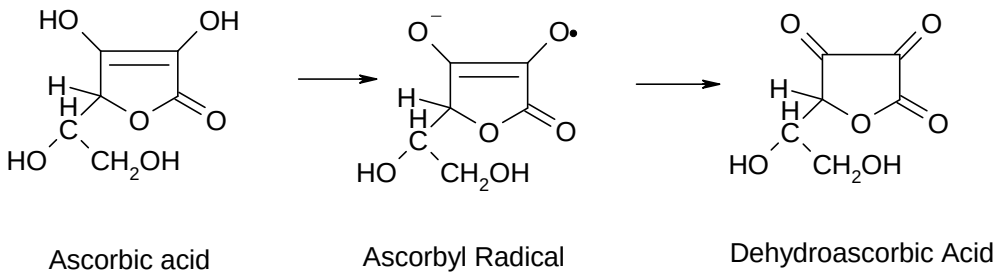

Fig. 7 – The oxidation scheme of the ascorbic acid.[18]

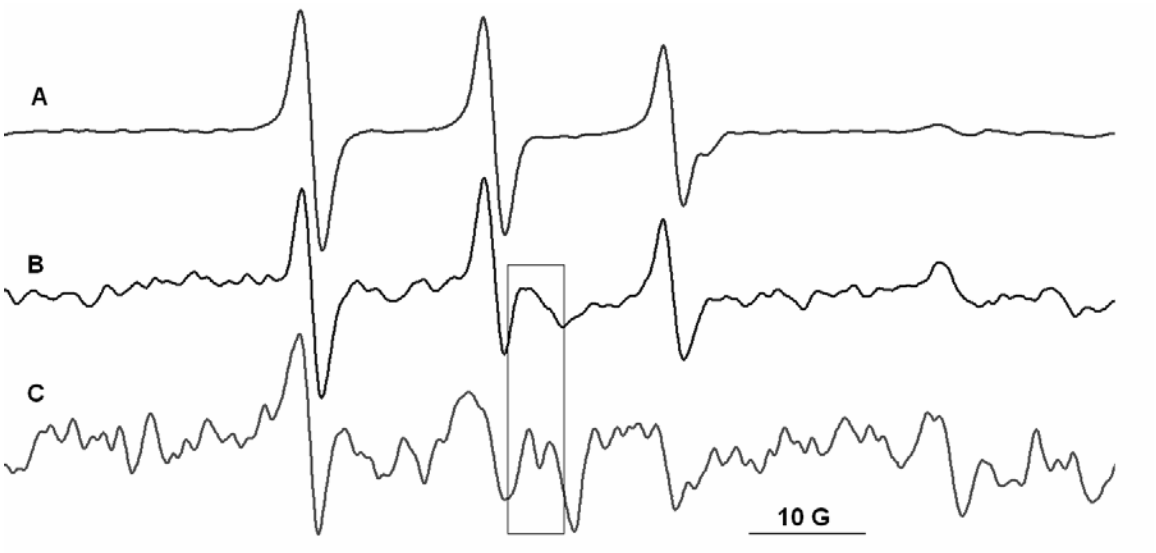

Fig. 8 – EPR spectra of TEMPO in dry extract
A) initial B) after 2 minutes, C) after 4 minutes, evidencing the presence of ascorbyl radical.

We aimed to prove that in the chloroplast obtained from leaves of *Rumex patientia* the ascorbyl radical is a part of antioxidative defense systems. Isolated chloroplasts from dried leaves of *Rumex patientia* plants, unexposed to ozone, are more efficient in reduction of TEMPO. In the same time, this extract has a higher content of ascorbic acid which allowed evidencing the formation of the intermediary ascorbyl radical (Fig. 8 B and C). In the frame is highlighted the presence of this radical characterized by the hyperfine splitting of a_H = 1.8 G.

The ascorbate from chloroplast extract reacts with radicalic species leading to reduced forms of dehydroascorbic acid.

EXPERIMENTAL

Materials

2[N-morpholino]-ethanesulfonic acid (MES), sorbitol, 5,5-dimethyl-1-pyrroline N-oxide (DMPO), 2,2,6,6-tetramethylpiperidine (TEMP) were obtained from Sigma-Aldrich.

Rumex patientia plants were collected from fields around Bucharest and transferred into several pots.

Chloroplast extraction

A number of eight *Rumex patientia* plants with similar phenotypical aspects were selected. Half of them were kept in normal atmosphere, while the other half were exposed to high concentration of ozone generated by a commercial instrument. The plants were exposed 8 hours daily to the ozone action in 8 days interval.

Chloroplasts from *Rumex patientia* were isolated by gradient centrifugation using D-sorbitol, following similar procedure as reported in literature. [15] Typically, 8 g of *Rumex patientia* leaves were homogenized to a paste using a mortar and pestle. Then 16 mL of an ice-cold sorbitol solution (0.5 M) was added in 3-4 mL increments to the paste grinding it to a smooth pulp after each addition. Then the mixture was filtered through about eight layers of clean cheese cloth in a glass funnel into an iced test tube. The filtrate was centrifuged at low speed (200 rpm) for 5 minutes. Following this, the supernatant was separated and centrifuged again at high speed (2000 rpm) for 10 minutes. The pellet contained chloroplasts.[16]

For the SDS PAGE the samples containing chlorophyll were centrifuged and the supernatant was diluted 1:2 with loading buffer (final concentration of 62.5 mM TRIS-HCl pH=7.9, 2.5% SDS, 0.002% Bromphenol Blue, 0.7135 M

(5%) β-mercapthoethanol, 10% glycerol). The proteins were denatured by heating samples for 10 minutes at 95ºC.

Reducing SDS-PAGE was run in 12% acrylamide gel using a Protean II Biorad gel-electrophoresis system. [17-18]

The EPR spectra were recorded at room temperature on a Jeol JES FA 100 spectrometer with the general settings as follows: center field 3026 G, sweep field 80 G, frequency 100 kHz, gain in the range 100 – 400, sweep time 30 s, time constant 0.1 s, modulation width 1 G, microwave power 1 mW.

Samples for EPR measurements were prepared using the re-suspended pellets (obtained from exposed or unexposed plants to ozone) in buffer solution B3 pH = 6.3 (50mM of MES, 15mM sodium chloride, 5mM magnesium chloride).[19] Depending on the experiments to this solution was added either a volume of 0.2 mL solution of DMPO (0.1M) prepared in B3 buffer or 0.2 mL of TEMP. The solution was fast transferred into glass capillary for EPR measurement.

CONCLUSIONS

To summarize, in this study it has been shown that the reactive oxygen species generated during the exposure of *Rumex patientia* plants to the ozone enriched atmosphere annihilate the antioxidative system of chloroplasts. The presence of oxygen singlet specie present in chloroplasts after exposure to ozone was evidenced by the increase EPR signal of TEMPO after mixing TEMP with the plant extract. The formation of DMPOX spin trap adduct reveal the presence of other ROS like $O_2^{\cdot -}$ or $HO^{\cdot}$.

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