Oxidative stress studies on plant DNA exposed to ozone

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This work is centered on the study of the behaviour of the plant DNA under conditions of oxidative degradation by ozone. The total DNA of high purity was extracted from fresh sunflower leaves. Using a system of free radicals generated *in vitro*, the action of reactive oxygen species on DNA was tested by combining spectrophotometrical technique with chemiluminescent assay and Polymerase Chain Reaction (PCR). The ozone and the reactive oxygen species have a high potential to interact with DNA molecules resulting in structural modifications and possible denaturations seriously affecting the cell function.

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1. Introduction

In the last decade, the oxidative stress (OS) effects on living systems have attracted much more attention [1-4].

Ozone (O_3), an allotrope of oxygen, is a powerful oxidizing agent that can induce OS. Ozone (O_3) is a minor constituent of Earth's atmosphere, which is continuously formed at altitudes above 30 km (stratosphere) by molecular oxygen (O_2) photodissociation in the presence of solar UV radiation with wavelengths below 242 nm [5]. The formed atomic oxygen is interacting with molecular oxygen, thus resulting ozone. In small quantities, ozone has beneficial effects on human health, but in certain doses there is a real risk due to its strong oxidizing properties being involved in the development of various respiratory diseases (e.g. asthma, choking) and lung cancer [6].

The high oxidation potential of ozone is mainly exploited for degradation of organic compounds and pharmaceuticals in water and wastewater [7].

Living cells, including plant cells, are continuously affected by increased levels of ozone. The normal metabolic processes are altered under these conditions, resulting in appearance of many compounds that reduce the intrinsic qualities of plants, including the antioxidant properties [8]. Similar to other gases, ozone enters plants through the leaves, by diffusion, causing the formation of reactive oxygen species that damage the vegetal cells. Biological impact of ozone on plants consists in reduced photosynthetic capacity, physiological and growth disturbances, senescence, premature death, reduced crop production, resulting in significant economic losses [9-15]. In agriculture, there is of great interest to obtain genotypes resistant to ozone, to improve quality and production of crops [3]. Herbal medicine uses the plants for treating various diseases caused by oxidative stress [16] and is therefore important to investigate the plant DNA and its components, which are target molecules for free radical attack [17, 18].

Deoxyribonucleic acid (DNA) is a long biopolymer resulted from polymerization of four types of deoxyribonucleotides - molecules that are made up of a sugar (deoxyribose), a phosphoric acid residue and a nitrogen base (Figure 1). DNA is an informational biomolecule, able to carry the genetic information stored in the sequence of nitrogenous bases [19] from one generation to another. DNA has a right-handed double helix structure [20]. The macromolecule of DNA is composed of two complementary and antiparallely polynucleotide chains held together by hydrogen bonds between complementary base pairs.

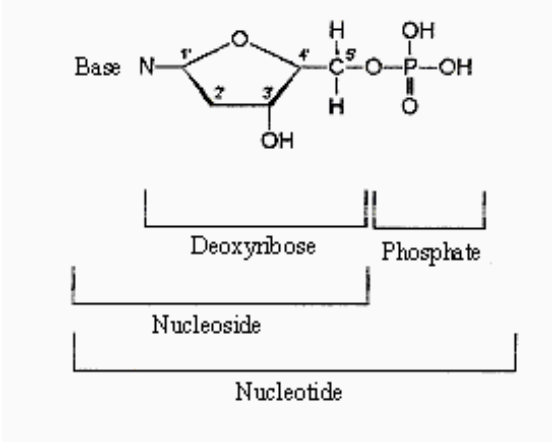


Fig. 1. The schematic structure of deoxyribonucleotides – the DNA building blocks.

DNA is one of the targets of oxidative stress. Ozone has been found to be mutagenic because its action is directed specifically toward the DNA biomolecules, being used as a sterilizing agent in food industry and in drinking water treatment to kill bacteria [21].

The reactive oxygen species attack not only the nucleobases, but also the sugar-phosphate DNA backbone causing DNA damage and breakage which are genotoxic and induce mutations [22].

Many studies show that ozone induces modifications in DNA structure, by breaking the DNA backbone [6].

In the present paper we have investigated the ozonated sunflower DNA by using several techniques (UV spectroscopy, chemiluminescence assay, electrophoretic analysis) in order to study the modifications occurred under oxidative stress conditions.

2. Experimental part

2.1. Reagents

The chemicals used for DNA preparation: cetyltrimethylammonium bromide (CTAB), Proteinase K, Ribonuclease A (RNase A), NaCl, boric acid, sodium bisulphite, hydroxymethylaminomethane base (Tris), ethylenediaminetetraacetic acid (EDTA) and for electrophoretic analysis: agarose (molecular biology grade), bromophenol blue (BPB), glycerol, ethidium bromide (EtBr) were purchased from Sigma Aldrich (Germany).

The chemicals used for chemiluminescence assay: Luminol (5-amino-2,3-dihydro-phthalazine-1,4-dione) and hydrogen peroxide were supplied from Merck (Germany).

The electrophoresis markers (1 kb DNA ladder and λ phage DNA) were obtained from Gibco BRL and the primers (10-mer oligonucleotides) from University British Columbia (UBC).

Solvents of analytical grade used in DNA extraction protocol (chloroform, isoamyl alcohol, 70 % ethanol, absolute ethanol, isopropanol) were purchased from Merck (Germany).

2.2. Methods and procedures

2.2.1 Extraction of DNA from sunflower leaves. The total genomic DNA was isolated from fresh cultivated Romanian sunflower (*Helianthus annuus* L.) leaves, an adaptation of the method described by Gentzbittel *et al.* [23]. The sunflower plants were in the stage of 3-4 pairs of leaves. Over about 5 grams of chopped plant tissue in liquid nitrogen, 9 mL CTAB buffer containing sodium bisulphite (25 mg/mL) were added to avoid the oxidation of the plant powder obtained. Samples were incubated for 30 min at 65°C, in the water bath. After cooling, it was added 5 mL mixture of chloroform: isoamyl alcohol (24:1) then the mixture was centrifuged (SIGMA 2-16 K) for 10 min at a speed of 10,000 rpm and the supernatant was recovered. Elimination of RNA was achieved by the addition of RNase A (10 mg/mL), mixing and incubation for 30 min at 37°C. Deproteinization was made by the addition of Proteinase K (20 mg/mL), mixing and incubation for 60 min at 37°C. DNA was then precipitated according to the following steps: 1) addition of 1 mL CTAB buffer preheated at 65°C and 2) addition of 7 mL mixture of chloroform: isoamyl alcohol (24:1), homogenization and centrifugation for 15 min at 10,000 rpm. The supernatant was mixed with 6 mL of cold 95 % ethanol, followed by a centrifugation for 10 min at 12,000 rpm. The sediment was dissolved in 1 mL of 1× TE buffer, then 600 μL mixture of chloroform:isoamyl alcohol (24:1) was added and centrifuged for 10 min at 13,000 rpm. In the supernatant 1M NaCl and 1 mL isopropanol have been added, followed by a centrifugation for 10 min at 13,000 rpm, with the removal of supernatant. The pellet was vacuum dried and dissolved in 1 mL of TE buffer pH 8.0 (10 mM Tris·HCl; 1 mM EDTA) and stored at -20°C before use.

DNA samples were divided into four vials and subjected to ozonation for different exposure times.

2.2.2 DNA quantitation

The concentration of the template-DNA has a very important role in PCR (*Polymerase Chain Reaction*). A combination of spectrophotometric, fluorometric and electrophoretic analyses was used in order to determine the exact DNA concentration and to check the purity degree of the samples.

- *DNA spectrophotometric quantitation*. Electronic absorption spectra of DNA samples were obtained on a double beam UV-VIS spectrophotometer Lambda 2S Perkin Elmer (PECSS software), in the wavelength range of 220-400 nm.
- *DNA fluorometric quantitation* is based on the highly specific binding of Hoechst dye (bisbenzimidazole) (Sigma-Aldrich) to the DNA using a Hoefer DNA Fluorometer after calibrating the apparatus with a standard solution of calf thymus DNA (Sigma-Aldrich). Thus, DNA samples were adjusted to 10 ng/μL in TE buffer (pH 8.0).
- *DNA electrophoretic quantitation*. The DNA concentration was also estimated by running the samples on 1 % agarose gel; the electrophoretic band intensities of the samples were compared with that of a standard: double-stranded bacteriophage *lambda* DNA. This method allows determining whether DNA is damaged and can make corrections to the values obtained by fluorometric dosage.

2.2.3 Sunflower DNA amplification

Polymerase chain reaction (PCR) is an *in vitro* reaction which involves many times replication of a specific fragment of DNA (target region). PCR has large applications in plant breeding to check plant resistance to biotic and abiotic stress.

The DNA amplification reactions were carried out in a final volume of 25 μL. The reaction mixture contains: Taq DNA polymerase buffer (1.5 mM), primer (0.5 μM), dNTPs (200 μM), 1.2 U Taq DNA polymerase (Appligene), 30 ng of genomic DNA (namely 3 μL of DNA samples that were previously adjusted to 10 ng/μL).

The reaction tubes were overlaid by mineral oil and placed in a MJR Research Thermocycler programmed for PCR reactions as follows: an initial step at 94 °C (4 min) for a complete DNA strand separation and Taq activation, followed by 35 cycles of amplification: 1 min at 94 °C (DNA "melting"), 1 min at 38 °C (annealing primers), 1 min at 72 °C (extension) and an end extension step at 72 °C (6 min). Finally, the PCR-tubes were kept at 4 °C holding temperature.

Amplified DNA fragments were separated by electrophoresis on 1.6 % (w/v) TBE (Tris-borate-EDTA) 0.5× agarose gel (TBE 1×contains 10 mM Tris, 8.9 mM boric acid and 2 mM Na_2EDTA). After staining with 0.2 μg/mL EtBr solution for 10 minutes, the gels were photographed with the imaging system Bioprint (Vilbert Lourmat) by illuminating the gel under 312 nm UV light (UV transilluminator).

2.2.4 The ozonation system

Ozone was produced by the system represented in Fig. 2. The presence of two rotameters (3) aims to control the dose and the flow rate of O_3. Also, installation is provided with vessels for O_3 dosage (4), for the absorption-emission of ozone (5) and for sampling of residual O_3 (6). Peristaltic pump (7) brings H_2O_2 into the reaction vessel (8). The ozonation system has a pH meter (9) and a conductometer (10). The pressure control of the solution above the oxidation column is assured by a manometer (11). Ozone was produced by passing oxygen through the generator and retained in acid solution because these solutions are stable for several hours. The ozone in generator passed through a solution of 1 mM perchloric acid (Sigma-Aldrich) for 20 min. A mixture containing ozone solution was transferred into a quartz cell in which was added 1 mM perchloric acid solution to a final volume of 2 mL. Ozone concentration was determined spectrophotometrically by recording the absorption maximum at 260 nm wavelength using the molar absorption coefficient of 3,300 $cm^{-1}M^{-1}$ for O_3 [24].

Ozonation conditions were: air flow rate: 80 L/h; ozone dose: 18 L/h; flow of the collected ozonated air: 10 mL/min = 0.18 mg O_3/min.

2.2.5 Chemiluminescence method

The chemiluminescence experiments were performed on a Chemiluminometer Turner Design TD 20/20, USA. Luminol has been used as a light amplifier which emits light when it is converted into an excited aminophtalate ion in the presence of reactive oxygen species. In this study the following chemiluminescence generator has been used: luminol (10^{-5} M) and hydrogen peroxide (10^{-5} M) in TRIS-HCl buffer solution pH 8.6. The antioxidant activity (percentage of scavenging of free radicals) of samples was calculated as described in [1].

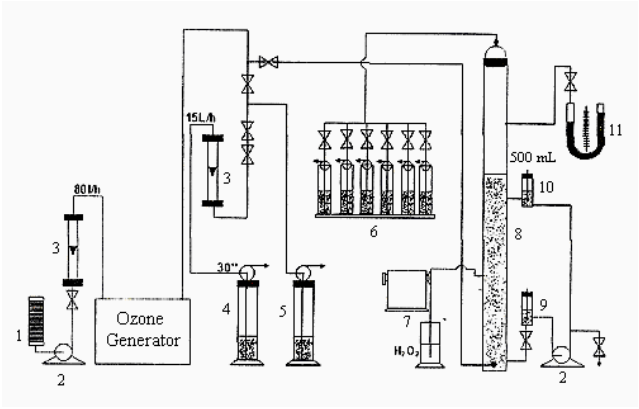


Fig. 2. Schematic representation of the ozonation system. 1 - Air dryer, 2 - recirculation pump, 3 - rotameters, 4 - ozone absorption vessel (for dosage), 5 - ozone absorption vessel (for the excess of ozone); 6 - battery residual ozone absorption vessels, 7 - peristaltic pump, 8 - oxidation column, 9 - pH meter, 10 - conductometer, 11 – manometer.

3. Results and discussions

3.1 Characterization of DNA samples by UV absorption spectroscopy

Useful information on DNA structure and analytical control of DNA extract can be provided by UV absorption spectrophotometric analysis which is an indispensable tool in nucleic acid research.

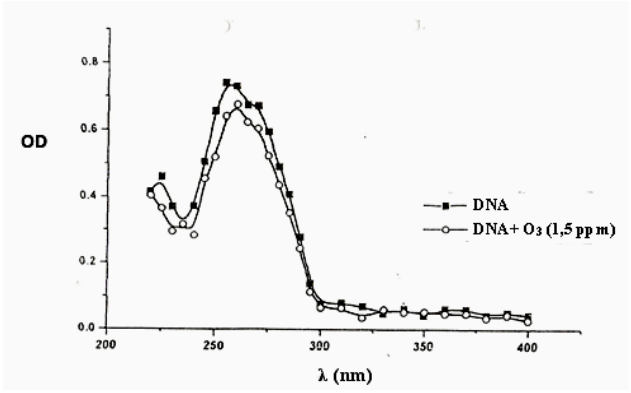
Solutions of DNA absorb the UV radiations in the wavelength range between 250 and 280 nm with a maximum at 260 nm, due to presence in the DNA molecule of purine and pyrimidine bases, the chromophores of nucleic acids, which have heterocyclic structures with conjugated double bonds.

DNA samples were quantified by measuring the optical densities at 260 nm assuming that one OD_{260} unit of double-stranded DNA (in a 1 cm pathlength cuvette) contains 50 ng/μL [25].

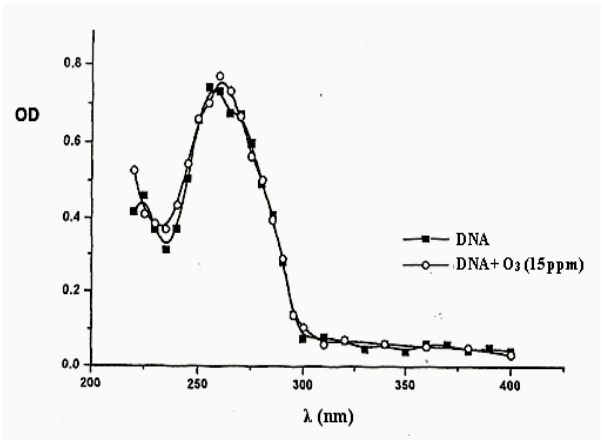
UV absorption spectroscopy also allows the study of DNA molecule distortions and of the transition: dsDNA→ssDNA. Thus, the double-stranded DNA (dsDNA) has a low absorption (hypochromism) because the chromophores stacking inside the double helix of the DNA macromolecule. On the contrary, denatured DNA (ssDNA) presents a hyperchromic phenomenon, namely a stronger absorption than native DNA, due to breaking of the hydrogen bridges between purine and pyrimidine bases [19].

The spectral changes of DNA samples before and after ozonation are displayed in Figure 3.

A characteristic band at 260 nm is observed which is assigned to the electronic transition, $\pi \rightarrow \pi^*$, coming from the aromatic structures of nucleotide bases.



a)



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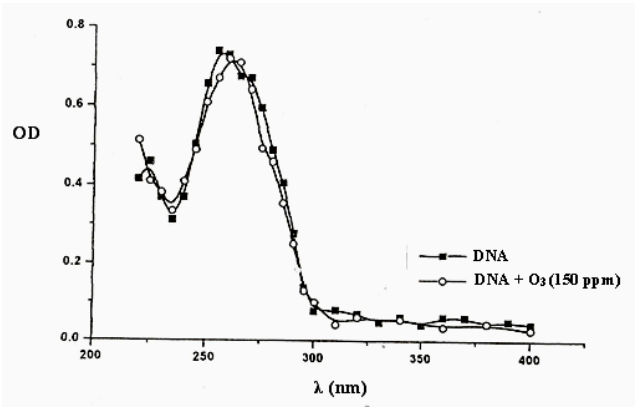

c)

Fig. 3. UV absorption spectra of DNA before and after ozonation with the following O_3 doses: 1.5 ppm (a), 15 ppm (b) and 150 ppm (c)

The ozonated samples showed a diminution of UV absorption at 260 nm. This hypochromic effect may be explained by the changing of DNA tertiary structure. The alteration of DNA is dependent on the ozone exposure time, longer treatment inducing more changes in macromolecular architecture.

These changes can be attributed firstly to the formation of peroxo-compounds of nucleotide components which are responsibles for three-dimensional reorganization of DNA architecture (see Fig. 3 a, c, hypochromic effect) and secondly to hydrogen bonds disruption between some bases (especially adenine - thymine hydrogen bridges) leading to a hyperchromic effect (Fig. 3b).

In order to identify the potential contaminants in the nucleic acid samples, some spectral criteria were monitored. The DNA samples obtained were of high purity (OD_{260}/OD_{235} = 1.72 and OD_{260}/OD_{280} = 1.8).

It is known from scientific literature [26] that:

- A DNA sample is pure if the optical density ratio OD_{260}/OD_{235} > 1.5 and the ratio OD_{260}/OD_{280} is in the range 1.7-2.0.
- Low OD_{260}/OD_{280} ratio are typically due to the presence of proteins, phenols or surfactants.
- Low values of OD_{260}/OD_{235} ratio indicate the presence of ethanol which was not completely removed in the DNA extraction process.

3.2 The oxidative stress behaviour of plant DNA

The plant DNA samples were subjected to an oxidative stress simulated *in vitro* using the luminol chemiluminescence (CL) assay.

The presence of a molecule in this system can produce an increase or a diminution of CL signal; this modification is correlated with the amount of free radicals.

Time evolution of CL signal shows that the addition of DNA biomolecules in the reaction mixture resulted in an increase in chemiluminescence intensity (Fig. 4). The ozonated DNA amplifies the CL signal stronger than unozonated DNA, since the ozonation leads to an increase in the amount of free radicals in the system, resulting in DNA damage.

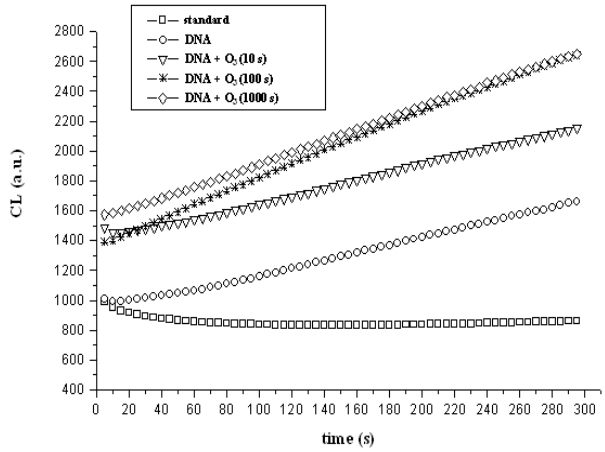

Fig. 4. Time evolution of CL signal of DNA (100 ng/μL) exposed to ozone

The oxidative DNA degradation could produce peroxide structures, mainly thymine peroxides, step preceding DNA chain breaking. The aggressivity of the ozone is also illustrated in Fig. 5. The analysis by chemiluminescence of the ozonated DNA samples shows a pronounced diminution of the antioxidative activity of the DNA in relation with the ozonation time. This CL

diminution points to structural modifications which were also put in evidence by the UV absorption spectra.

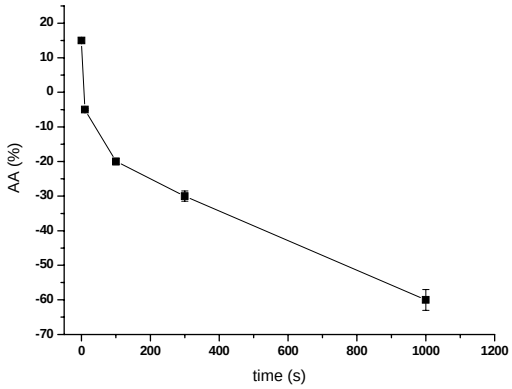

Fig. 5. Antioxidant activity evolution of sunflower DNA (100 ng/μL) subjected to ozonation for different O_3 exposure times

The alteration of DNA primary structure during the oxidative stress leads to inevitable changes of the plant rate growth and to a diminution of its resistance to diseases, features that can be evaluated *in vitro* by the apparition of the pro-oxidative character of DNA samples.

3.3 Characterization of oxidative damaged DNA by electrophoresis analysis

Analysis of electrophoretic migration of total DNA (unozonated and ozonated) provides some qualitative information about the samples (Fig. 6). It is noted that a higher dose of ozone (150 ppm) induces DNA structural changes, revealed by smeared bands on the gel (lanes 3 and 5).

The purity degree of the samples was quite high; it is observed a total elimination of RNA and a good deproteinisation (Fig. 6).

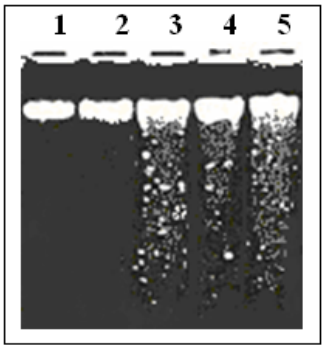

Fig. 6. Electrophoregram of the total DNA (ozonated and unozonated). Standard: λ DNA (10 ng/μL)

Legend of Figure 6:

Lane	*1*	*2*	*3*	*4*	*5*
Sample	*λ DNA*	*Total DNA unozonated*	*Total DNA ozonated with 1.5 ppm O_3*	*Total DNA ozonated with 15 ppm O_3*	*Total DNA ozonated with 150 ppm O_3*

The PCR amplification experiments were performed using 10-mer primers with high GC content, resulting in more stable DNA/primer hybrids.

The electrophoregram of the PCR products reveals a good yield of amplification for unozonated DNA (Fig. 7, lanes 2 and 6) and for ozonated DNA exposed to 15 ppm O_3 (Fig. 7, lane 4). For the other doses of ozone, the yield of amplification was lower (Fig. 7, lanes 3, 5, 10).

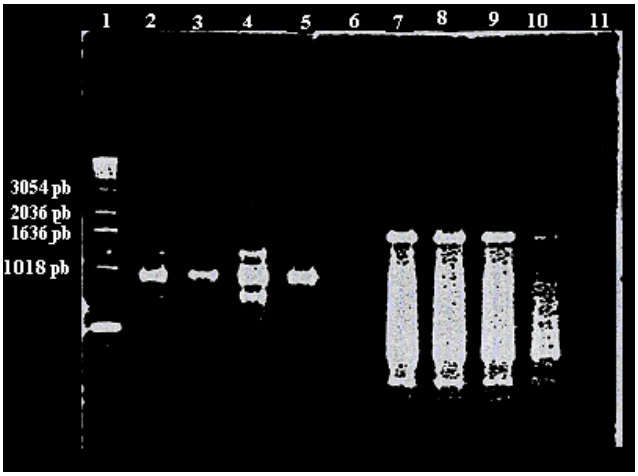

Fig. 7. Electrophoregram of the PCR products using the primers 357 (AGGCCAAATG), the columns 2÷6 and 375 (CCGGACACGA), the columns 7÷14. Size marker: 1Kb DNA Ladder.

Legend of Figure 7:

Lane	1	2	3	4	5	6
Sample	1Kb DNA Ladder	DNA unozonized	DNA-1.5 ppm O_3	DNA-15 ppm O_3	DNA-150 ppm O_3	Blank (the reaction mixture without DNA)
Lane	7	8	9	10	11	
Sample	DNA unozonized	DNA-1.5 ppm O_3	DNA-15 ppm O_3	DNA-150 ppm O_3	Blank (the reaction mixture without DNA)	

The electrophoregram of the PCR products using the primer 357 (AGGCCAAATG) reveals the presence of a DNA band of about 1,000 bp in size for all of the samples, but in the case of the ozonated DNA with 15 ppm O_3 dose it is observed the apparition of two DNA bands (~1,200 bp and ~900 bp) which indicates that this dose of ozone caused significant degradation in the DNA structure.

4. Conclusions

In the present paper the effect of ozone on plant DNA has been investigated by combining spectral techniques with chemiluminescence method and electrophoretic analysis.

The ozonation of sunflower DNA samples lead to modifications in the structure of this biomolecule. The ozonation of DNA induced changes in the structural

architecture of the biomacromolecule evidenced by hyperchromic or hypochromic effect in the UV absorption spectra.

DNA ozonation also resulted in elevated chemiluminescent signals which are strongly related to a decrease of plant resistance to oxidative stress. The chemiluminescence data were sustained by the UV absorption spectra.

Electrophoretic study of plant DNA samples subjected to ozonation revealed a slightly reduction of PCR amplification of DNA due to its distortion and the possible formation of peroxo-compounds.

Studies of oxidative stress on DNA extracted from crop plants are of great interest to agriculture, to find and create new plant varieties resistant to various oxidizing agents (e.g. O_3).

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