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EFFECT OF TROPOSPHERIC OZONE ON TWO WHITE CLOVER (*TRIFOLIUM REPENS* L. CV. 'REGAL') CLONES WITH DIFFERENT OZONE SENSITIVITY EXPOSED AT RURAL AREA OF WIELKOPOLSKA REGION

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Abstract: In this paper, we present results indicating ozone effect on visible plants response as well as on other parameters, such as dry weight, chlorophyll concentration, cell membrane stability and salicylic acid content in bioindicator plants. Ozone-resistant and -sensitive clones of white clover (*Trifolium repens* L. cv. 'Regal') were used in the investigations. The experiment was carried out in ambient air conditions of the Wielkopolska province (Poland) in 2005 growing season. The exposure led to changes in the level of plant response parameters that might be used as potential biomarkers of oxidative stress triggered by tropospheric ozone in ambient air conditions.

INTRODUCTION

Tropospheric ozone is the most important secondary air pollutant influencing plants negatively [16, 25]. Natural processes, as well as anthropogenic pollutants, increase ozone concentration in the troposphere. Among natural sources of pollutants the most important are lightning discharges and vertical ozone transport from the stratosphere. However, it should be pointed out that natural processes in the balance of tropospheric ozone in urbanized areas have a significantly lower impact than human activity, since the main source of ozone in the troposphere is a sequence of photochemical reactions of nitrogen oxides in the presence of hydrocarbons [7, 22] whose initial concentration has recently drastically increased as a result of human activity. The emission of ozone precursors has decreased in Europe during last two decades, owing to the increased use of catalytic converters in cars [11]. Although this reduction has resulted in a decline of ozone maximum concentrations, the annual mean concentrations are still increasing [8, 11], perhaps because of transport of ozone and its precursors from remote distances. Some European tropospheric ozone comes from North America and Asia [9]. The negative effect of tropospheric ozone on environment can be observed [8, 26].

Some studies have revealed that bioindicator plants are very useful for the determination of air quality in areas where automatic pollutant detection cannot be applied [20]. At the end of the 1980s, the International Cooperation Program of the influence of pollutants on natural and crop plants (ICP Vegetation) was started, and its principles and regulations cover both Europe and the USA. Today 28 countries participate in this program, which operates as part of the convention on long-range transboundary air pollution [18]. Investigations carried out within the framework of the project are also included in recommendations of the European Union Directive [10]. The program focuses on the analysis of losses caused by ozone among crop plants and its effects on grasslands and wetlands. Sites with bioindicator plants are frequently located in European countries in places distant from direct sources of air pollutants. One of the bioindicators is white clover (*Trifolium repens* L. cv. 'Regal') whose two clones reveal different tropospheric ozone sensitivities [18].

Ozone transport from the atmosphere into the internal leaf spaces occurs mainly via stomata. However, ozone molecules can interact with the leaf surface causing cuticle damage [22]. Inside plant cells, ozone-derived reactive oxygen species (ROS) lead to plasma membrane, chloroplast and mitochondrial injuries reducing photosynthesis and biomass production. Ozone symptoms can vary depending on plant species [14]. Characteristic changes appear in sensitive plants as slight discolorations or necrotic lesions of white, black or brown colour on the upper leaf surface.

Ozone can negatively affect photosynthesis process. It has been proved that ozone causes injuries and inhibition at almost every stage of photosynthesis, starting from light absorption to starch accumulation [13]. The above injuries manifest themselves in the reduction of chlorophyll concentration, increase of pigment fluorescence and changes in excitation transfer [13, 33].

The phytotoxicity of ozone results from the fact that, after penetrating into leaf apoplast, it can directly react with lipids and proteins of the cell membrane. Such reactions, as well as ozone decomposition in the cell aqueous environment, lead to the generation of reactive oxygen species and organic radicals [12] that can further react with proteins, DNA and lipids, influencing the permeability of membranes, reducing photosynthesis and progressing plant aging [12, 31]. Ozone itself and the excess of ozone-derived ROS lead to the appearance of oxidative stress inside plant cells [2, 5] which, in turn, induces a complex biochemical mechanism of ROS excess removal. Salicylic acid plays a crucial role in the regulation of the plant response to stress factors, including the oxidative stress caused by ozone. Its biosynthesis can be induced directly by an excess of ROS, as well as by ethylene. The regulatory function of this compound comes from its ability to alter the activity of antioxidative enzymes which can lead to ROS accumulation and suicidal death of plant cells. In leaves not exposed to a stress factor, salicylic acid participates in the formation of a number of protective compounds involved in the non-specific defense mechanisms [31].

In this paper we have the following objectives: (i) to examine if there are differences between responses of two white clover clones with different ozone sensitivity exposed to ambient air conditions, (ii) to determine if one of the presented parameters could be a marker of ozone stress in ambient air conditions, (iii) to determine if these clones could be a good ozone bioindicators at rural area of Wielkopolska region.

MATERIALS AND METHODS

Experimental design

Two clones of white clover (*Trifolium repens* L. cv. 'Regal') with different sensitivities to ozone were used. Seedlings of both clones were obtained from the chief coordinator of the ICP Vegetation program. Plants were exposed to ozone under natural conditions according to the methodology of this program [16, 19].

Exposures (10 series) were carried out in the growing season (from May to the end of September) 2005. Some of the results of 2005 are related to these obtained in 2004, and already published [6]. During the experiment the same plants, earlier cultivated in greenhouse conditions for four weeks, were exposed to ambient air. Each exposure series lasted four weeks after which visible injuries were estimated and the above-ground plant parts were cut and prepared for additional analyses. To avoid a four-week gap between series, the second series of plants (B series) overlapped series A by two weeks (Tab. 1).

Table 1. Dates of white clover exposure series (harvest)

Exposure series number	Terms of exposure/harvest of white clover
1A	30.04 – 30.05.2005
1B	14.05 – 14.06.2005
2A	30.05 – 28.06.2005
2B	14.06 – 12.07.2005
3A	28.06 – 26.07.2005
3B	12.07 – 09.08.2005
4A	26.07 – 23.08.2005
4B	09.08 – 06.09.2005
5A	23.08 – 20.09.2005
5B	06.09 – 04.10.2005

Plants were cultivated in 20 liter pots with standard peat and sand mixture. Continuous water supply was provided by fiber glass wicks dipped in tray with water placed under the pots.

Plants were exposed to field condition in the Tarnowo Podgórne area, about 15 km west of the City of Poznan, Poland (N 52°27'43,65"; E 16°41'17,44"). As recommended by the ICP Vegetation program, the exposure site was located at least 200 m from the main road and 50 m from buildings.

Each series consisted of 30 plants (15 of each clone) and 2 plants of each clone were placed in a controlled greenhouse conditions. Moreover, as far as possible similar growth conditions to ambient were provided.

Following the recommendations of the ICP Vegetation methodology, the degree of ozone injury in sensitive clones and the dry weight of entire plants were determined.

A 7-degree scale was used to estimate ozone-caused injury of clover leaves: 0 – no injuries, 1 – very small injuries, appearance of the first symptoms, 2 – small injuries (1–5% of leaves affected), 3 – medium injuries (5–25% of injured leaves), 4 – intensive injuries (25–50% of injured leaves), 5 – very intensive injures (50–90% of injured leaves), 6 – total damage (90–100% of leaves with heavy injuries) [19].

Bioindication results of our experiment were compared to air pollution concentration measured by automatic monitoring system of Wielkopolska Regional Inspectorate for Environmental Protection. The ozone concentration was presented as AOT 40, which is the sum of values above 40 $nL \cdot L^{-1}$ recognized as minimum concentration of ozone which affects plants negatively. This is connected with the fact that plants remain in the environment all the time and the ozone impact is cumulative. For our purposes we calculated AOT 40 values for each exposure series (28 days) during daylight hours, as did Fumagilli *et al*. [16] and Postiglione *et al.* [28] in their studies.

Plant material analyses

After the exposure, the dry weight content (obtained with a dryer at 65°C) of whole plants (shoots, leaves and flowers) and the degree of negative ozone effect on cropping of white clover were determined. Dry weight content, presented as the mean value (in grams per pot) for the sensitive and resistant clone for each exposure series, was measured. In addition, the dry weight content of leaves was also determined after drying at 105°C.

After each exposure series chlorophyll, salicylic acid content and cell membrane stability were determined in leaves of both clones.

Chlorophyll in leaves was determined after extraction with dimethyl sulfoxide; the absorbance of extracts was measured according to the method of Shoaf and Lium [32]. Chlorophyll *a+b* concentration was calculated with Arnon's formula and presented per 1 gram of dry weight because of the appearance of necrosis on leaves of the sensitive clone. This solution was suggested by Adedipe *et al.* [1] and Fletcher *et al.* [15].

The cell membrane stability was determined with the conductivity method proposed by Bandurska and Gniazdowska-Skoczek [4]. The 2 cm^2 samples of leaf were treated by 10 mL of deionized water and kept for 24 hours in 10°C. Afterwards, they were washed three times in deionized water and kept again for 24 hours in 10°C. Then warmed to room temperature and electrical conductivity was measured. Following the conductivity leaves were autoclaved for 15 minutes (temperature 105°C and pressure 0.5 atmosphere) and then cooled to room temperature. Electrical conductivity was measured again. The cell membrane stability is presented in the percentage of cell membrane injuries according to the following equation: $I = [1 - (1 - T_1/T_2)/(1 - C_1/C_2)] \bullet 100$, where I – percentage of cell membrane injury, T_1 and C_1 – first conductivity measurement of exposed and controlled sample, T_2 and C_2 – second conductivity measurement of exposed and controlled sample of leaves.

Salicylic acid in a free form (SA), as well as total salicylic acid (TSA), were extracted from plant leaves according to the methodology recommended by Yalpani *et al.* [34]. Three samples were taken from each plant, and three plants of each white clover clone were employed in every harvest series. Salicylic acid content in leaves was analyzed with a WATERS Company chromatograph (Milford, Ma, USA) composed of 2699 Separation Module Alliance and 2475 Multi-λ Fluorescence Detector. Chromatographic separations were performed on a Spherisorb ODS2 WATERS Company column (3 μm, 4.6 × 10 mm). The content of the salicylic acid released from its glycoside (SAG) was calculated as the difference between assays without and with glycoside enzymatic degradation (SAG = TSA – SA). The recovery of the standard added to samples amounted to 89% for SA and 86% for TSA assays.

Statistical analysis

The data were analyzed using the statistical software STATISTICA 8.1. Results were analyzed with a factorial ANOVA (two- or three-way) with harvest, clone and place of exposure fixed factors. The Tukey's test was employed to analyze the detailed differences between measured parameters and graphically presented in this paper.

RESULTS AND DISCUSSION

Ozone concentration in ambient air and its relationship to visible leaf injury of sensitive white clover clone

Ambient air ozone concentration during the growing period was at a level causing visible leaf injuries to the white clover sensitive clone. The daily average ozone concentration in exposure series was between 17.2 and 31.3 $nL \cdot L^{-1}$. During the experiment AOT 40 values for one month exposure series fluctuated between 449 and 2007 $nL \cdot L^{-1}$. The total value for the entire research period was comparable to the value registered in 2002, when intensive ozone injuries in tobacco plants were observed in the City of Poznan and the surrounding areas [35]. The highest level of the pollutant was observed during the first exposure series (Tab. 2).

Table 2. Ozone concentration presented in AOT 40 unit, mean daily ozone concentration and visible leaves injury of sensitive white clover clone presented in 1–6 scale

Exposure series	AOT40 [$nL \cdot L^{-1}$]	Mean daily ozone concentration [$nL \cdot L^{-1}$]	Mean values of sensitive leaves injuries (in 1–6 scale)
1A	2007	30.4	1.8
1B	1643	30.1	1.4
2A	1055	30.1	1.5
2B	1394.5	31.3	1.7
3A	960	27.7	1.1
3B	681	24.5	0.7
4A	499.2	22.1	1.0
4B	728.8	22.5	1.2
5A	963.2	22.5	1.4
5B	694.5	17.2	0.7
Average	5484.4/5141.8*	25.8	1.25

* summarized value of A series/B series

The observed ozone concentration was comparable to that of other countries participating in the ICP Vegetation program in 2005, mainly southern Germany and Slovenia. Definitively lower concentrations were observed in Great Britain and Sweden [18]. Other air pollutants were at levels which did not exceed limited values determined by EU Directive 2008/50/EC [10]. Moreover, the concentrations were several times lower than limited values, both for human health and plant risk. Annual mean value of SO_2 was at the level of 0.5 $\mu g \cdot m^{-3}$ (limited value for plant's risk – 20 $\mu g \cdot m^{-3}$) and NO_x at the level of 4.3 (limited value for plant's risk – 30 $\mu g \cdot m^{-3}$). Daily mean value of SO_2 for the whole presented

experimental period was at the level of 1.88 $\mu g \cdot m^{-3}$ (limited value for human health is 125 $\mu g \cdot m^{-3}$). Hence, these air pollutants probably did not affect significantly exposed plants.

In the ozone-sensitive clone of white clover, visible symptoms of ozone effect appeared as necrosis lesions between leaf veins. Ozone injuries were not observed in plants of the resistant clone, owing to moderate ozone concentrations in the ambient air. The average visible necrosis caused by ozone observed on leaves of sensitive plants ranged from 0.7–1.8 (in the 0–6 scale). The most severe injuries were observed for the 1A exposure series (30.04–30.05), the slightest for 3A (12.07–09.08) and 5B (06.09–04.10), i.e. at the end of the growing season (Tab. 2).

The analysis of the linear regression for the ozone injuries of sensitive white clover clone and the ozone concentration, expressed as AOT 40, showed a statistically significant positive correlation at the level $\alpha = 0.05$ (Fig. 1). A similar tendency was observed in white clover experiment in 2004, however a lack of significance level was then observed, $p = 0.053$ [6].

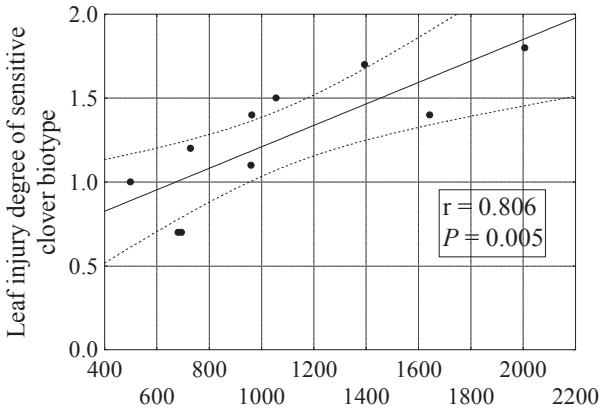

Fig. 1. Correlation between ozone concentration (AOT 40) and leaf visible injuries of white clover ozone-sensitive clone

Dry weight, chlorophyll concentration and cell membrane stability

The mean value of the whole plant dry weight was between 28.4 and 431 g per pot in the sensitive clone and from 17.7 up to 430.2 g in the resistant one, while in the control it ranged from 57.9–275.9 g and 14.3–152.0 g per pot of sensitive and resistant clones, respectively. Factorial ANOVA revealed the influence of harvest, clone and site of exposure/cultivation at high significant level for dry weight concentration in whole white clover (Tab. 3).

For each exposure series, the whole plant dry weight and ozone concentrations presented as AOT 40 demonstrated negative correlation and were not statistically significant at the level $\alpha = 0.05$ (Fig. 2a and 2b).

The correlation coefficient was also negative for both clones in an experiment conducted in 2004, and significance was at the level $p = 0.190$ and $p = 0.180$ for sensitive and resistant clone, respectively [6]. Dry weight of white clover leaves was higher for the ozone-resistant clone than for the sensitive one for both the exposed and control series. This was also valid for results obtained in 2004 [6]. Similar results were observed during

Table 3. Factorial ANOVA (two- or three-way) of selected parameters with harvest, clone and site fixing factors (*** p < 0.001; **p < 0.01; *p < 0.05; ns – not significant)

Parameter	Harvest/series	Clone	Exposure/ cultivation site	Interaction
Cell membrane injury	***	***	-	ns
Chlorophyll *a*+*b* content	***	ns	***	***
Dry weight content in leaves	***	***	ns	**
Dry weight content in whole plants	***	***	***	***
Free salicylic acid	***	***	*	*
Glycoside salicylic acid	***	**	*	*

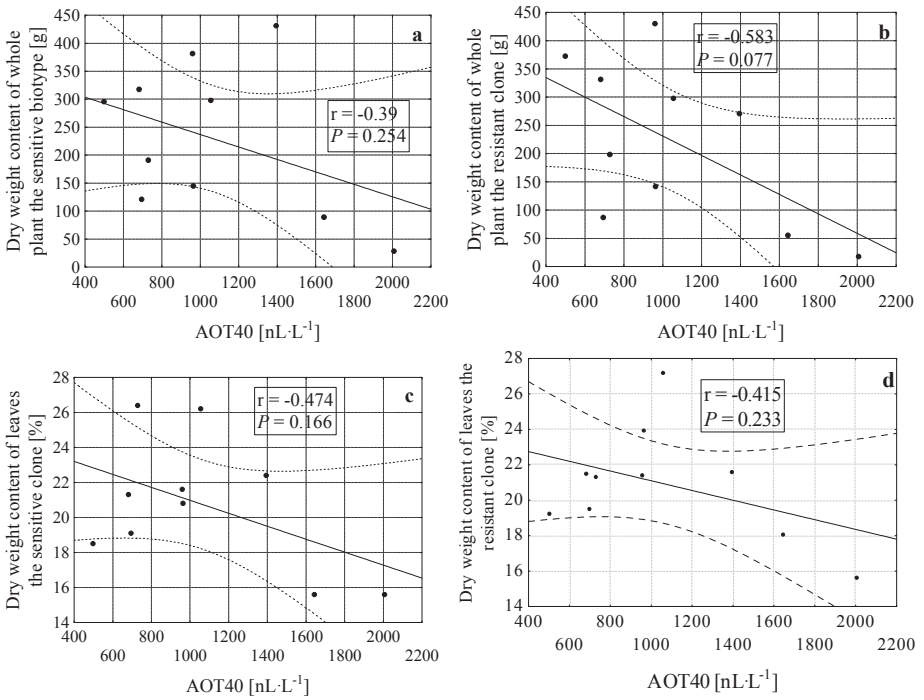

Fig. 2. Correlations between AOT 40 and:
a – whole plant dry weight of sensitive clone [g]
b – whole plant dry weight of resistant clone [g]
c – leaves dry weight of sensitive clone [%]
d – leaves dry weight of resistant clone [%]

an investigation conducted in the Mediterranean region [28]. Linear regression for the dry weight of leaves of both white clover clones was negative and not statistically significant (Fig. 2c and 2d). The factorial ANOVA revealed high significant influence of harvest and clone to dry weight concentration in white clover leaves (Tab. 3).

Ozone interacts with plants indirectly by triggering the exaggerated accumulation of reactive oxygen species [31] which can further react with proteins and lipids of mem-

branes causing changes in their permeability and leakage of electrolytes, particularly of such important ions as potassium [22], and injuring canals of the Ca^{2+} [3]. Therefore, it is crucial to assess the cytoplasmic membrane permeability of indicator plants. Throughout the entire research period the mean value of cell membrane stability of white clover fluctuated between 8.2 and 24.6% for the sensitive clone and between 6.3 and 10.4% for the resistant one. The analysis of variance revealed high significant influence of harvest and clone effect on cell membrane stability (Tab. 3). More severe cell membrane injuries were observed for the sensitive clone than for the resistant one (Fig. 3). Similar results were obtained in the case of tobacco plants of two cultivars with different sensitivity to tropospheric ozone [35]. Therefore, it can be presumed that the assessment of cell membrane injuries facilitates the identification of the plant response and sensitivity to ozone as well as the resulting oxidative stress in plant cells.

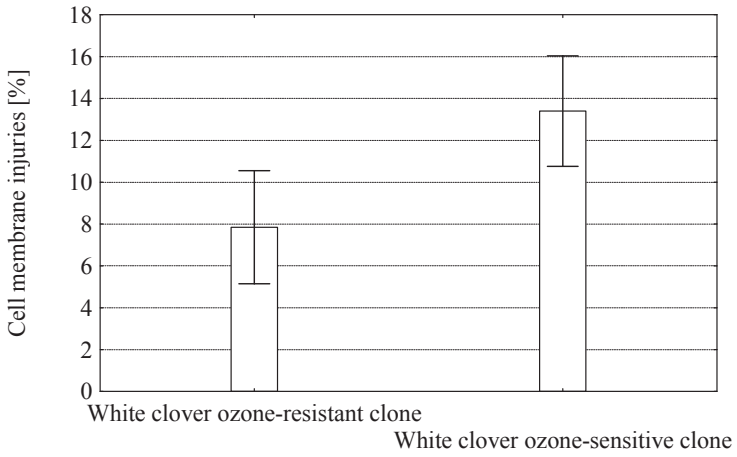

Fig. 3. Means and 0.95 confidence intervals of cell membrane injuries degree [%] both white clover clones

Throughout the entire research season, the concentration of chlorophyll *a*+*b* in the dry matter of leaves after exposure to ambient ozone fluctuated in the range from 2.66 to 5.61 mg g^{-1} for the sensitive clone and from 2.06 to 6.07 mg g^{-1} for the resistant one. In the case of control plants of both clones, this value was assessed at the levels between 3.83 and 7.02 mg g^{-1}, and from 3.54 up to 8.68 mg g^{-1}, respectively. The chlorophyll concentration did not depend on clone effect, but on harvest and exposure/cultivation site effects (Tab. 3).

The chlorophyll *a*+*b* concentration in control plants of both clones was significantly higher ($\alpha = 0.05$) than in plants exposed to ambient air conditions. The difference of pigment concentration between sites reached 20% in the case of sensitive clone, and 30.8% in the resistant one (Fig. 4).

As previously described [21, 24], chronic exposure to ozone can decrease pigment concentration in plant leaves. Experiments carried out under ambient air conditions are essential not only to determine the mechanism of plant response to tropospheric ozone, but also in order to find biomarkers that could be used in a wide scale experiments. So far, only a few experiments focused on determining the relation between pigment ac-

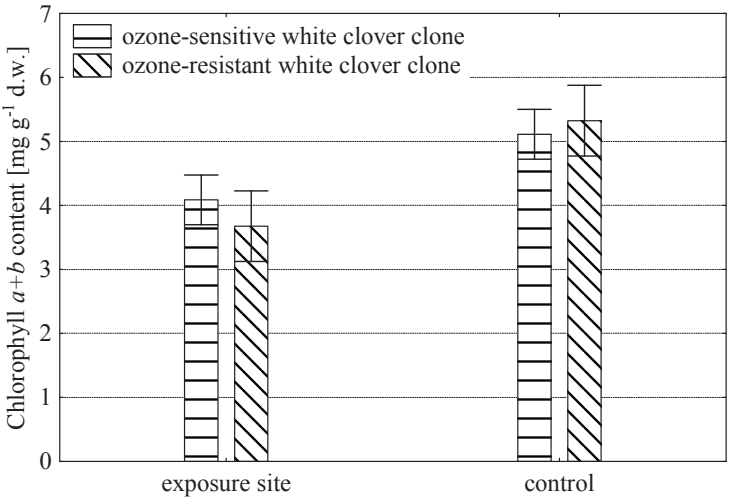

Fig. 4. Means and 0.95 confidence intervals of chlorophyll *a* + *b* content in dry mass of both clones at exposition and control site

cumulation and ozone level have been carried out for ambient air [23]. The majority of investigations were conducted in controlled conditions (fumigation chambers) [17, 27, 29, 30]. In the presented studies, exposition of plants to ambient air conditions (including tropospheric ozone) caused reduction of chlorophyll *a*+*b* concentration in white clover of both clones. Hence, this parameter can be considered instead of the assessment of visible leaf injury to determine the intensity of the oxidative stress caused by tropospheric ozone in ambient air conditions.

Salicylic acid content

Changes of antioxidant activity require programmed changes within plant cells in which salicylic acid has probably a crucial regulatory function, as proved previously in the case of alfalfa (*Medicago sativa* L.) [5].

Free and conjugated salicylic acids were determined for white clover leaves exposed to tropospheric ozone under ambient conditions in nine series during the growing season. The average contents of free salicylic acid after exposure varied from 146.3 up to 708.1 ng g f.w.$^{-1}$ for the sensitive clone and from 45.8 to 654.7 ng g f.w.$^{-1}$ for the resistant one. At the control site, SA concentration in leaves fluctuated from 151.1 up to 686.2 ng g f.w.$^{-1}$ and from 84.5 to 386.1 ng g f.w.$^{-1}$, respectively, for the investigated clones. Moreover, the average SA content for the exposed plants decreased by 9.7% in the case of the ozone sensitive clone and rose by 6.4% for the resistant one in comparison to the control plants of both clones (Fig. 5a).

The factorial ANOVA revealed a high influence of harvest and clone effect on SA content in white clover, while for SAG only harvest effect influenced at high statistical level (Tab. 3).

The average content of SAG after exposure was determined at the levels from 836.9 to 5386.7 ng g f.w.$^{-1}$ for the ozone sensitive clone and from 248.6 to 5711.1 ng g f.w.$^{-1}$ - for the resistant one. In the case of the control plants of both clones, this value fluctu-

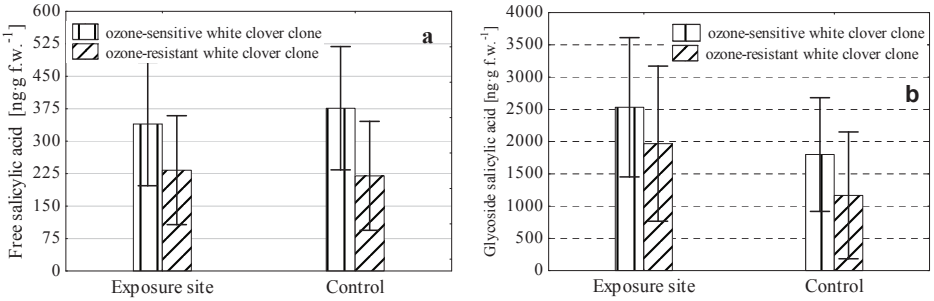

Fig. 5. Means and 0.95 confidence intervals SA (a) and SAG (b) both clones at exposure and control site

ated within the range from 938.2 to 2892 ng g f.w.$^{-1}$ and from 736.6 to 2131.9 ng g f.w.$^{-1}$, respectively (Tab. 3).

Leaves of the white clover ozone-sensitive clone synthesized higher amounts of SAG than the resistant ones at both the exposure and control sites (Fig. 5b). The increase of SAG content after exposure reached approximately 41.1% for the sensitive clone, and 53.1% for the resistant clone in comparison to the control plants of both clones.

The accumulation of the salicylic acid was observed in tobacco plants (*Nicotiana tabacum* L.) treated with ozone under ambient conditions in earlier investigations. The biosynthesis of this phenylpropanoid regulatory compound and the accompanying plant reaction and programmed cell death were closely related to the degree of ozone presence in the troposphere [31]. The analysis of salicylic acid in leaves of white clover showed higher concentrations of free salicylic acid and its glycoside in the sensitive clone than in the resistant one. Nevertheless, the higher increase of salicylic acid content was observed for its conjugated form, i.e. salicylic acid glycoside, suggesting its potential role in oxidative stress measurement. Moreover, the intensive accumulation of salicylic acid in leaves of the resistant white clover clone can suggest that it could be a good biomarker of ozone stress even in cases where visible injuries are scarcely manifested.

CONCLUSIONS

An ozone-sensitive clone of white clover revealed positive correlation of leaf injury with tropospheric ozone concentration to such an extent that it could be applied as an indicator of air pollution with ozone under ambient air conditions in rural areas of the Wielkopolska province.

The cell membrane injuries were more severe for the sensitive clone of white clover than for the resistant one and can serve as an indicator of plant/crop injuries triggered by tropospheric ozone. A negative relation (but not significant) was also found for dry weight of both clones and ozone concentration in the ambient air.

The chlorophyll *a*+*b* concentration in exposed white clover was lower than in the control plants. Moreover, the lower chlorophyll concentration in the case of the resistant clone shows its potential use as an ozone stress biomarker in the ambient air conditions.

We also observed the enhanced biosynthesis of salicylic acid in exposed white clover leaves, both in free as well as in conjugated form, i.e. salicylic acid glycoside. There-

fore, it is quite possible to use this compound as an indicator of plant oxidative stress caused by ozone, even when there is a lack of visible symptoms.

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WPŁYW OZONU TROPOSFERYCZNEGO NA DWA BIOTYPY KONICZYNY BIAŁEJ (*TRIFOLIUM REPENS* L. CV. 'REGAL') O RÓŻNYM STOPNIU WRAŻLIWOŚCI NA OZON EKSPONOWANYCH NA TERENIE ROLNICZYM WIELKOPOLSKI

W pracy przedstawiono rezultaty badań wpływu ozonu troposferycznego na widoczną reakcję roślin, jak również na inne parametry, tj. poziom biomasy, stężenie chlorofilu, stabilność błon cytoplazmatycznych i zawartość kwasu salicylowego. W badaniach zastosowano rośliny bioindykacyjne, tj. wrażliwe i odporne na ozon biotypy koniczyny białej (*Trifolium repens* L. cv. 'Regal'). Rośliny eksponowano na stanowisku pozamiejskim w sezonie wegetacyjnym 2005 roku na terenie Wielkopolski. Badania wykazały różnice w poziomach badanych parametrów roślin eksponowanych i kontrolnych ze wskazaniem potencjalnych markerów stresu ozonowego roślin w warunkach polowych.