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# SALICYLIC ACID – A POTENTIAL BIOMARKER OF TOBACCO BEL-W3 CELL DEATH DEVELOPED AS A RESPONSE TO GROUND LEVEL OZONE UNDER AMBIENT CONDITIONS

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Salicylic acid content and benzoic acid 2-hydroxylase (BA2H) activity were investigated in tobacco Bel-W3 and Bel-B leaves after exposure to tropospheric ozone in the conditions of ambient air. Plants were exposed in accordance with a standard methodology for ozone biomonitoring, in a three-year experiment. Free salicylic acid (SA), conjugated with glucose (SAG), and as a product of the BA2H activity was quantified with HPLC. In order to evaluate ozone injuries of leaves, an open source image analysis software was employed. Plants exposure to ambient ozone resulted in enhanced BA2H activity and intensified salicylic acid biosynthesis in leaves of Bel-W3 cultivar showing visible ozone injuries. The BA2H activity significantly correlated with SAG for ozone-exposed Bel-W3 plants. Both injuries and salicylic acid biosynthesis rate depended on the growth phase of leaves and nearly linear correlation between SA content and injuries was found for particular leaves of Bel-W3.

*Keywords:* Necrotic lesions – oxidative stress – salicylic acid – tobacco Bel-W3 – tropospheric ozone

## INTRODUCTION

Tropospheric (ground level) ozone has been posing a serious threat to plants since first observations of ozone-caused injuries of crops in the 1950s in the United States [31, 52]. Ozone concentration in the troposphere has increased fourfold since the beginning of the industrial era and its peak values in the most industrialised countries attain 100 to 400 ppb [21]. Its proliferation and continually growing concentrations make ozone one of the constituents of the ambient air causing considerable devastation among wild-growing plants, reducing annual tree increments and negatively influencing species biodiversity [53]. In addition, ozone reduces the yield of sensitive crops and decreases their commercial value [1, 2, 30]. Mean ozone concentrations in Europe and North America during summer months continue to grow (by

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0.2–1% annually) and are sufficient to cause damage to ozone sensitive plants [11, 13, 19, 45, 51].

Acute exposures of plants to ozone at concentrations ranging from 120 to 500 ppb extending over several hours lead to damages of the cell membrane and cell death as the result of the extreme disturbance in ion fluxes. Sub-acute and chronic exposures (maximum daily concentration from 40 to 120 ppb for several days during the vegetative season) lead to controlled plant response, recently reviewed by Castagna and Ranieri [4], including both aging and programmed cell death (PCD) similar to that of a plant hypersensitive response (HR) to pathogens [36, 37, 48]. Investigations with ozone-sensitive plants aim at determining intracellular stress indicators whose level, alongside values of visible injuries, might inform about the plant's physiological response to ozone [6, 16, 49].

Oxidative stress is accompanied by an increased biosynthesis of salicylic acid (o-hydroxybenzoic acid) as confirmed in many plant species in case of pathogen attack [10, 32, 55] as well as in plants treated with ozone in controlled conditions [24, 33, 35]. Salicylic acid (SA) influences many physiological and metabolic processes regulating seed germination, growth of the root system and leaves, and chlorophyll biosynthesis, as well as flowering and thermogenesis [34, 41, 42]. Moreover, salicylic acid plays regulatory roles in plant resistance to pathogens and, probably, to other factors causing oxidative stress (ozone, xenobiotics, heavy metals, etc.) [15, 42]. SA alters hydrogen peroxide metabolism in infected plants by intensifying SOD activity and inactivating CAT and APx, thus leading to the accumulation of hydrogen peroxide which oxidises cell constituents, reduces the efficiency of photosynthesis and destroys cell membranes leading to the cell death [7, 40].

The aim of the present investigations was to evaluate the impact of ambient ozone on the biosynthesis of salicylic acid in leaves of two tobacco cultivars showing diverse sensitivity to ozone (Bel-W3 – sensitive, and Bel-B – resistant), and to provide information about the usefulness of this compound as a biomarker of ozone stress in conditions of ozone biomonitoring. Our studies on salicylic acid biosynthesis will provide novel information about plant physiological response to ozone under complex ambient conditions to support *in vitro* studies and fully controlled experiments. In our experiment tobacco plants were exposed to ambient air of the city of Poznan and surrounding rural areas. Poznan is located in west-central Poland, on the Warta river with predominant western winds. Nowadays, Poznan is Poland's fifth largest city and fourth largest industrial centre with a population of over half a million inhabitants, and the area of 261 $km^2$. In Poland, ozone biomonitoring using tobacco plants have been conducted so far in Krakow and its surroundings as well as in the Carpathian Mountains, and the results of these studies confirmed the ozone negative impact on flora in that country [3, 14, 29].

## MATERIALS AND METHODS

### *Tobacco plants growth and exposure to ambient ozone*

Tobacco (*Nicotiana tabacum* L.) plants of Bel-W3 and Bel-B cultivars were cultivated and exposed to the tropospheric ozone in conditions of ambient air in accordance with the standard methodology VDI 2000 [50] applied in the biomonitoring program of air contamination with ozone carried out in European countries [23].

Seeds of both cultivars were sown into the substrate which consisted of a mixture of Stender E910 soil and washed quartz sand (8:1, w/w), and contained slowly released fertilizers meeting the plant's nutrient requirement throughout the cultivation in a greenhouse and exposure in the field. Five plants of Bel-W3 and one plant of Bel-B cultivar were placed at each exposure site. Each exposure set was placed on a steel rack 90 cm above the ground level. A synthetic fibre net of 50% shading coefficient covering the top part of the rack protected plants against excessive insolation. A continuous water supply was guaranteed by glass fibre wicks placed in a tray with water directly under the container.

Two-week exposures of tobacco plants were carried out from the end of May until the beginning of September during the vegetative seasons of 2004–2006. In the first two years of experiment, plants were exposed at one site in a suburban area about 10 km from Poznan (Tarnowo Podgorne), whereas during the last year of investigations

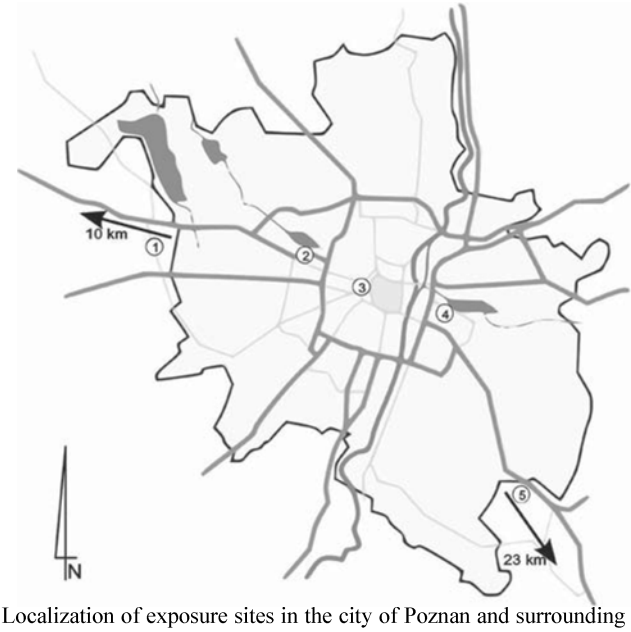


*Fig. 1.* Localization of exposure sites in the city of Poznan and surrounding rural areas (1 – Tarnowo Podgorne, 2 – Botanical Garden, 3 – Kaponiera Round, 4 – Polanka, 5 – Zaniemysl)

the number of sites was increased to five, three of which were within the boundaries of the city of Poznan, while the remaining two sites were in rural areas (Fig. 1). Control plants remained in the greenhouse during the entire exposure period. Exposure sites were selected according to the EuroBionet project suggestions, as well as on the basis of previous investigations conducted in this area [20] to achieve a wide range of ozone-caused injury. Exposure site located in Botanical Garden of Adam Mickiewicz University (No. 2) was equipped with an automatic monitoring of air contamination and meteorological parameters, provided by The Voivodship Inspectorate for Environmental Protection (WIOS) in Poznan.

### *Computer analysis of visible ozone injuries*

Leaves of the Bel-W3 plants showing ozone-induced necrotic lesions (starting from the fifth leaf counted from the plant base) were cut, and after instantaneous scanning with an optic scanner were frozen in liquid nitrogen and stored at –45 °C till later analyses. Because of the absence of ozone injuries, leaves of the Bel-B cultivar as well as leaves of control plants of both cultivars were frozen in liquid nitrogen without prior scanning. Only leaves free from any mechanical damage were collected.

In order to ensure objective evaluation of the leaf injuries (as a percent of the total leaf surface) caused by tropospheric ozone, we employed an open source image manipulation and analysis software. The total leaf surface was determined by use of the GIMP software, whereas the surface of ozone-induced necrotic lesions was assessed with software for graphics structural analysis – IMAGEJ. These programmes were used instead of visual assessment applied in biomonitoring programs based on a 5-point scale of the Leaf Injury Index (LII) [18, 23]. The graphic image analysis we used made it possible to count up lesions with the surface exceeding 0.22 $mm^2$.

### *Analysis of salicylic acid content*

Salicylic acid in free form (SA), as well as that conjugated as a glucoside (SAG), were determined according to the methodology recommended by Yalpani et al. [55]. Tobacco leaves were ground in liquid nitrogen to a fine powder from which approximately 0.50 g was taken for analysis. Salicylic acid was extracted twice with methanol. After centrifugation, the supernatant was divided into two equal parts and the solvent was evaporated to dryness under a stream of nitrogen. A 5% solution of trichloroacetic acid was added to one part and then SA was extracted three times into ethyl acetate:cyclopentane:isopropanol (100 : 99 : 1, v/v/v). In order to determine the total (free and glucoside bound) salicylic acid (TSA), 40 units of β-glucosidase (SIGMA) in acetate buffer (0.1 M, pH 5.2) were added to the second part of the dry extract and incubated for 90 minutes at 37 °C. The reaction was terminated by the addition of 5% trichloroacetic acid and then salicylic acid was extracted as described

above. After solvent evaporation, the dry residue was dissolved in a mobile phase (0.2 M acetate buffer, pH 5.0; 0.5 mM EDTA) and analysed by the HPLC method coupled with fluorometric detection with a WATERS Company chromatograph (Milford, Ma, USA) composed of 2699 Separation Module Alliance and 2475 Multi-λ Fluorescence Detector. Chromatographic separation was on a Spherisorb ODS2 WATERS Company column (3 μm, 4.6 × 10 mm). Detection parameters were as follows: 295 nm for excitation and 405 nm for emission. The content of the salicylic acid released from its glucoside was calculated as the difference between assays without and with glucoside enzymatic degradation (SAG = TSA – SA). The recovery of the standard added to samples amounted to 89% for SA determination and 86% for TSA assays.

## *Analysis of benzoic acid 2-hydroxylase activity*

The BA2H activity was determined in accordance with the method described by Leon et al. [26]. The activity of the enzyme was described as the number of nanomoles of salicylic acid obtained as a reaction product during one hour for the enzyme extracted from 1 gram fresh weight of tissue [nmol SA (g FW h)$^{-1}$]. Salicylic acid was determined by the HPLC method as described above.

Approximately 0.30 g of the homogenized tissue was taken for the analysis. The enzyme was extracted with HEPES buffer (SIGMA) (20 mM, pH 7.0) containing 12.5 mM 2-mercaptomethanol, 10 mM sorbitol, 1% PVP and 1 mM PMSF. The reaction mixture of 500 μl total volume consisted of 10 μmol of HEPES buffer (pH 7.0), 1 μmol of benzoic acid, 1 μmol of NAD(P)H (SIGMA), 100 μl of the extraction buffer and 100 μl of the extract. The mixture was incubated at 30 °C for 30 minutes. The reaction was terminated by the addition of 15% trichloroacetic acid and salicylic acid was extracted three times with an organic mixture (see: the analysis of SA content). After solvent evaporation, the dry residue was transferred into the mobile phase and analysed with the HPLC method as described above.

## *Statistical analysis*

The statistical analysis of the data was performed with the assistance of the Statistica software, Open Office Calc and Microsoft Excel. Single- and two-factor analysis of variance was carried out employing the F-test. The Unequal N HSD test (modified Tukey's test) was performed in the case of two-factor ANOVA and the Student's *t*-test for MANOVA analysis of variance. The significance of the relation between variables was described with the assistance of Pearson's correlation coefficient. The analysis of variance for the linear regression was performed with the F-test.

## RESULTS

### *One-site exposure/multi-leaf analysis*

The exposure of tobacco plants to ambient ozone resulted in elevated concentrations of salicylic acid both in free and bound forms compared with control plants of both cultivars. This increase was only slight in plants of the resistant cultivar, but quite conspicuous in the case of the ozone-sensitive one. Simultaneously, SA amounted to a lower percentage of TSA in Bel-W3 plants following their exposure to tropospheric ozone. The BA2H activity determined in tobacco leaves of the Bel-W3 cultivar was higher in plants exposed to ozone than in control plants of this cultivar (Table 1). The two-factor analysis of variance for groups of unequal N did not allow rejection of the zero hypothesis, both for the main effects (cultivar, exposure) and for the interaction of two factors with the probability of making an error of the first type at the level of 0.05 for the majority of the examined traits. However, it was demonstrated that the effect of the exposure differentiated significantly not only SA and TSA content, but also the activity of BA2H (Table 1).

Ozone-caused injuries were observed only for Bel-W3 plants exposed to ambient air conditions. Most frequently, they assumed the form of double-sided necroses with colours ranging from straw to dark brown, and less frequently of oily spots, each time situated between vascular bundles. Usually, ozone injuries covered uniformly the surface of middle leaves, i.e. 6th and 7th leaf counted from the plant base. In older leaves they could be found primarily at the leaf base, while in younger leaves at the peak. The mean value of injuries was slightly over 4%, while the maximum injury was only 27%, indicating that in years 2004/2005 the degree of air contamination with ozone at the exposure site was low. The highest values of injuries were found for the 6th leaf, and classified as the 5th degree in the 11-score Horsfall's and Cowling's injury scale (26–50%), whereas in the case of the consecutive leaves, i.e. 7th, 8th and 9th, the value of injuries decreased along with their chronological order (Table 2). From among 45 leaves analysed, 36 exhibited ozone injuries between the 1st and the 2nd degree of the same scale (1–6%). The mean number of necrotic lesions assumed the highest value for the 7th leaf and declined in the following order: leaves 8 > 6 > 5 > 9 (Table 2). The number of necrotic lesions increased with consecutive degrees of damage on the 11-point scale, reaching the highest value for the 3rd degree (7–12%) above which the number of lesions was observed to drop as a result of the increase in their diameter. Consequently, they tended to merge and form necrotic areas of large surface.

Leaf chronological order was found to differentiate significantly the contents of SA, SAG and TSA in the exposed plants of the Bel-W3 cultivar, whereas in the case of the Bel-B cultivar, the age of leaves failed to exert a significant influence on salicylic acid content. In the Bel-W3 cultivar, the highest SA content was in the 7th leaves, lower and comparable in leaves numbered as 6 and 8, and lowest in leaves 5 and 9. On the other hand, the content of SAG and TSA declined successively for leaves 6, 8 and 5 falling to its lowest value in the case of leaf 9. Mean BA2H activity

*Table 1*

Salicylic acid (SA – free salicylic acid, SAG – salicylic acid released from glucoside, TSA – total salicylic acid) content and the activity of benzoic acid 2-hydroxylase (BA2H) in tobacco leaves collected in one-site/multi-leaf experiment (in years 2004/2005)

| ANOVA effect | | Exposure | | | Cultivar | | Exposure×Cultivar | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Total | Exposed | Control | Bel-W3 | Bel-B | Bel-W3 | | Bel-B | |
| | | | | | | | Exposed | Control | Exposed | Control |
| Number of leaves analyzed | | 71 | 62 | 9 | 50 | 21 | 45 | 5 | 17 | 4 |
| SA [ng g$^{-1}$ FW] | av | 60.18 | 65.70 | 18.09 | 73.15 | 28.40 | 79.61 | 16.30 | 29.69 | 21.06 |
| | sd | 48.09 | 48.34 | 12.35 | 49.90 | 22.18 | 48.46 | 11.60 | 23.27 | 15.61 |
| | p | – | 0.0297 | | 0.1676 | | 0.0958 | | | |
| SAG [ng g$^{-1}$ FW] | av | 663.45 | 743.09 | 56.23 | 901.51 | 80.21 | 998.58 | 47.32 | 81.82 | 71.09 |
| | sd | 764.29 | 778.58 | 45.23 | 791.48 | 61.58 | 777.58 | 42.17 | 64.03 | 55.45 |
| | p | – | 0.0571 | | 0.0768 | | 0.0627 | | | |
| TSA [ng g$^{-1}$ FW] | av | 723.63 | 808.79 | 74.31 | 974.66 | 108.61 | 1078.19 | 63.60 | 111.51 | 92.14 |
| | sd | 794.25 | 806.90 | 47.93 | 818.71 | 60.85 | 800.34 | 46.71 | 63.04 | 53.98 |
| | p | – | 0.0472 | | 0.0710 | | 0.0559 | | | |
| SA in TSA [%] | av | 19.12 | 17.34 | 32.88 | 13.99 | 31.99 | 11.99 | 31.23 | 31.33 | 35.44 |
| | sd | 19.66 | 18.61 | 22.65 | 14.43 | 24.67 | 13.01 | 15.55 | 23.56 | 36.01 |
| | p | – | 0.0919 | | 0.0893 | | 0.2690 | | | |
| Number of leaves analyzed | | 40 | 35 | 5 | 40 | – | 35 | 5 | Not analyzed | |
| BA2H [nmol SA (g FW h)$^{-1}$] | av | 3.64 | 3.88 | 2.44 | 3.64 | – | 3.88 | 2.44 | | |
| | sd | 1.51 | 1.50 | 1.51 | 1.51 | – | 1.50 | 1.51 | | |
| | p | – | 0.0302 | | – | – | – | – | | |

ANOVA two-factor analysis of variance at $\alpha \leq 0.05$ (av – mean value, sd – standard deviation, p – empirical level of significance).

*Table 2*

Salicylic acid (SA – free salicylic acid, SAG – salicylic acid released from glucoside, TSA – total salicylic acid) content and the activity of benzoic acid 2-hydroxylase (BA2H) and injury for subsequent leaves of tobacco plants exposed to ambient ozone in one-site/multi-leaf experiment (in years 2004/2005)

| | | Bel-W3 | | | | | | Bel-B | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Total | Leaf number | | | | | Total | Leaf number | | | |
| | | | 5 | 6 | 7 | 8 | 9 | | 6 | 7 | 8 | 9 |
| Number of leaves analyzed | | 45 | 7 | 14 | 11 | 9 | 3 | 17 | 4 | 4 | 7 | 3 |
| SA [ng g$^{-1}$ FW] | av | 79.61 | 38.51 | 86.53 | 102.50 | 84.91 | 43.36 | 29.69 | 27.00 | 41.78 | 23.75 | 31.70 |
| | sd | 48.46 | 30.48 | 62.40 | 26.99 | 41.47 | 25.45 | 23.27 | 16.70 | 37.37 | 17.50 | 30.58 |
| | p | 0.0396 | | | | | | 0.6998 | | | | |
| SAG [ng g$^{-1}$ FW] | av | 998.58 | 756.31 | 979.33 | 1529.42 | 821.18 | 239.58 | 81.82 | 87.23 | 50.30 | 84.62 | 124.20 |
| | sd | 777.58 | 781.37 | 584.37 | 1006.10 | 520.25 | 202.50 | 64.03 | 115.01 | 45.84 | 44.20 | 12.42 |
| | p | 0.0487 | | | | | | 0.6388 | | | | |
| TSA [ng g$^{-1}$ FW] | av | 1078.19 | 794.82 | 1065.85 | 1631.92 | 906.09 | 282.94 | 111.51 | 114.23 | 92.09 | 108.37 | 155.91 |
| | sd | 800.34 | 804.16 | 590.87 | 1025.75 | 548.89 | 214.45 | 63.04 | 110.14 | 29.31 | 53.55 | 43.00 |
| | p | 0.0403 | | | | | | 0.7455 | | | | |
| Injury [%] | av | 4.316 | 3.696 | 6.233 | 4.757 | 2.609 | 0.327 | – | | | | |
| | sd | 5.315 | 5.049 | 7.879 | 3.371 | 1.162 | 0.180 | – | | | | |
| | p | 0.3385 | | | | | | – | | | | |
| Number of lesions | av | 302.82 | 149.43 | 293.14 | 470.82 | 320.22 | 37.67 | – | | | | |
| | sd | 253.91 | 150.24 | 215.55 | 284.74 | 257.26 | 35.02 | – | | | | |
| | p | 0.0220 | | | | | | – | | | | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SA/1% injury [ng (g FW %)$^{-1}$] | av | 36.68 | 30.69 | 19.79 | 33.59 | 37.86 | 137.27 | – | | | | |
| | sd | 38.80 | 31.32 | 13.97 | 31.10 | 22.76 | 62.41 | – | | | | |
| | p | 0.0000 | | | | | | – | | | | |
| SAG/1% injury [ng (g FW %)$^{-1}$] | av | 346.83 | 329.85 | 280.08 | 343.41 | 365.61 | 654.21 | – | | | | |
| | sd | 241.80 | 176.20 | 209.19 | 193.38 | 243.44 | 529.00 | – | | | | |
| | P | 0.1981 | | | | | | – | | | | |
| TSA/1% injury [ng (g FW %)$^{-1}$] | av | 383.51 | 360.53 | 299.87 | 377.00 | 403.47 | 791.48 | – | | | | |
| | sd | 253.82 | 192.10 | 214.21 | 191.73 | 260.61 | 467.10 | – | | | | |
| | p | 0.0431 | | | | | | – | | | | |
| Number of leaves analyzed | | 35 | 9 | 14 | 5 | 4 | 3 | Not analyzed | | | | |
| BA2H [nmol SA (g FW h)$^{-1}$] | av | 3.88 | 3.50 | 3.61 | 5.31 | 3.85 | 3.89 | | | | | |
| | sd | 1.50 | 0.58 | 1.30 | 2.62 | 1.33 | 0.98 | | | | | |
| | p | 0.2312 | | | | | | | | | | |
| BA2H/1% injury [nmol SA (g FW %)$^{-1}$] | av | 2.50 | 3.82 | 1.25 | 1.45 | 1.13 | 14.72 | | | | | |
| | sd | 3.81 | 5.17 | 0.75 | 1.13 | 0.99 | 3.63 | | | | | |
| | p | 0.0029 | | | | | | | | | | |

MANOVA analysis of variance at $\alpha \leq 0.05$ (av – mean value, sd – standard deviation, p – empirical level of significance).

*Table 3*

Correlation analysis of salicylic acid (SA – free salicylic acid, SAG – salicylic acid released from glucoside, TSA – total salicylic acid) content, the activity of benzoic acid 2-hydroxylase (BA2H) and visible ozone injury of tobacco Bel-W3 leaves at $\alpha \leq 0.05$

| One-site exposure/multi-leaf analysis (2004/2005) | | | | N | $av_{(X)}$ | $av_{(Y)}$ | r | $r_2$ | p |
|---|---|---|---|---|---|---|---|---|---|
| (1) | X | BA2H activity | [nmol SA (g FW h)$^{-1}$] | | | | | | |
| | Y | SA | [ng g$^{-1}$ FW] | 40 | 3.64 | 55.51 | 0.6434 | 0.4139 | 0.0000 |
| | | SAG | | 40 | 3.64 | 873.52 | 0.8280 | 0.6856 | 0.0000 |
| | | TSA | | 40 | 3.64 | 929.03 | 0.8266 | 0.6832 | 0.0000 |
| (2) | X | Injury | [%] | | | | | | |
| | Y | BA2H activity | [nmol SA (g FW h)$^{-1}$] | 40 | 4.09 | 3.64 | 0.3879 | 0.1505 | 0.3954 |
| | | SA | [ng g$^{-1}$ FW] | 50 | 3.96 | 73.72 | 0.5018 | 0.2518 | 0.3356 |
| | | SAG | | 50 | 3.96 | 917.58 | 0.4000 | 0.1600 | 0.3764 |
| | | TSA | | 50 | 3.96 | 991.29 | 0.4176 | 0.1744 | 0.3785 |
| (3) | X | Number of lesions | – | | | | | | |
| | Y | BA2H activity | [nmol SA (g FW h)$^{-1}$] | 40 | 283.14 | 3.64 | 0.6799 | 0.4623 | 0.0000 |
| | | SA | [ng g$^{-1}$ FW] | 50 | 277.58 | 73.72 | 0.4906 | 0.2407 | 0.0004 |
| | | SAG | | 50 | 277.58 | 991.29 | 0.8844 | 0.7821 | 0.0000 |
| | | TSA | | 50 | 277.58 | 917.58 | 0.8851 | 0.7834 | 0.0000 |
| Multi-site exposure/one-leaf analysis (2006) | | | | | | | | | |
| (4) | X | Injury | [%] | | | | | | |
| | Y | SA | [ng g$^{-1}$ FW] | 30 | 17.96 | 399.24 | 0.9568 | 0.9154 | 0.0000 |
| | | SAG | | 30 | 17.96 | 2687.45 | 0.9130 | 0.8335 | 0.0000 |
| | | TSA | | 30 | 17.96 | 3085.72 | 0.9258 | 0.8573 | 0.0000 |
| (5) | X | Number of lesions | – | | | | | | |
| | Y | SA | [ng g$^{-1}$ FW] | 30 | 230.33 | 399.24 | –0.1747 | 0.0305 | 0.3559 |
| | | SAG | | 30 | 230.33 | 2687.45 | 0.0029 | 0.0000 | 0.9879 |
| | | TSA | | 30 | 230.33 | 3085.72 | –0.0248 | 0.0006 | 0.8965 |

N – number of leaves analyzed; av – mean value; r – Pearson's linear correlation coefficient; $r^2$ – determination coefficient; p – empirical level of significance.

*Table 4*
Salicylic acid (SA – free salicylic acid, SAG – salicylic acid released from glucoside, TSA – total salicylic acid) content and injury of tobacco Bel-W3 leaves collected in multi-site exposure / one-leaf experiment (in year 2006)

| | SA [μg g$^{-1}$ FW] | | SAG [μg g$^{-1}$ FW] | | TSA [μg g$^{-1}$ FW] | | Injury [%] | | Number of lesions – | |
|---|---|---|---|---|---|---|---|---|---|---|
| | av | sd | av | sd | av | sd | av | sd | av | sd |
| *A. Exposure site* | | | | | | | | | | |
| Tarnowo Podgorne | 0.564 | 1.141 | 3.080 | 4.903 | 3.645 | 6.012 | 21.781 | 38.647 | 207.33 | 278.30 |
| Botanical Garden | 0.830 | 1.018 | 5.140 | 6.480 | 5.970 | 7.497 | 30.203 | 36.105 | 212.33 | 231.44 |
| Kaponiera Round | 0.023 | 0.010 | 0.132 | 0.072 | 0.156 | 0.082 | 2.041 | 1.833 | 84.16 | 95.76 |
| Polanka | 0.389 | 0.420 | 3.008 | 2.420 | 3.397 | 2.794 | 23.201 | 25.864 | 247.83 | 207.42 |
| Zaniemysl | 0.187 | 0.174 | 2.075 | 1.513 | 2.262 | 1.619 | 12.565 | 10.468 | 400.00 | 358.39 |
| Control | 0.008 | 0.004 | 0.005 | 0.003 | 0.013 | 0.006 | 0 | 0 | 0 | 0 |
| *B. Exposure series (2006) – without control site* | | | | | | | | | | |
| 29.05–11.06 | 0.049 | 0.039 | 0.571 | 0.731 | 0.620 | 0.768 | 3.609 | 5.150 | 129.40 | 137.30 |
| 12.06–25.06 | 0.308 | 0.279 | 3.211 | 1.804 | 3.520 | 1.988 | 15.516 | 13.875 | 483.80 | 359.41 |
| 26.06–09.07 | 1.331 | 1.313 | 7.546 | 6.566 | 8.878 | 7.812 | 55.185 | 45.809 | 98.20 | 199.00 |
| 24.07–06.08 | 0.584 | 0.627 | 3.771 | 4.049 | 4.356 | 4.666 | 24.693 | 17.753 | 137.60 | 97.87 |
| 07.08–20.08 | 0.095 | 0.056 | 0.853 | 0.387 | 0.949 | 0.435 | 8.400 | 2.074 | 486.00 | 138.31 |
| 21.08–04.09 | 0.024 | 0.011 | 0.169 | 0.066 | 0.194 | 0.069 | 0.747 | 0.389 | 47.00 | 29.57 |
| Mean | 0.399 | 0.722 | 2.687 | 3.078 | 3.085 | 3.601 | 17.958 | 26.744 | 230.33 | 234.27 |

av – mean value for whole exposure season(A)/all exposure sites (B), sd – standard deviation.

assumed the highest value for leaves designated as 7, while in the remaining cases the activity of this enzyme showed lower and similar values. The content of salicylic acid and BA2H activity falling to 1 percent of the observed injuries assumed similar values for leaves 5–8, and were distinctly higher for the youngest leaves, i.e. 9 (Table 2).

Correlation analysis revealed the existence of a strong direct proportional dependence between BA2H activity and SAG and TSA content and a slightly weaker one with SA. Ozone-induced injuries remained in the closest association with the SA content and BA2H activity, however these correlations were not significant. At the same time, a strong correlation was observed between the number of necrotic lesions and SAG and TSA content and a slightly weaker, but still significant with BA2H activity and SA content (Table 3).

### *Multi-site exposure/one-leaf analysis*

In the third year of the experiment, plants of the Bel-W3 and Bel-B cultivars were exposed to tropospheric ozone under natural conditions in five sites and in 6 series during the vegetative season. The analyses of the salicylic acid content and ozone-induced leaf injuries were carried out for leaves designated as 7 counted from the plant base.

A very strong positive linear correlation was observed between the content of salicylic acid, both SA and SAG, and ozone-induced injuries of leaves. The analysis of the relation between the salicylic acid content and the number of lesions in the multi-site approach failed to show any correlations between these traits over the entire range of injuries (Table 3).

Ozone-caused injuries occurred only on the Bel-W3 cultivar with values ranging from 0 to 100%, and with the mean slightly over 18%. The highest visible leaf injury was recorded for plants exposed from June 26th to July 9th at three out of five exposure sites, excluding the city centre (Kaponiera Round), and the most remote rural area (Zaniemysl). Simultaneously, salicylic acid content in leaves of Bel-W3 cultivar shared the same pattern as ozone-caused injuries considering sites and terms of exposure (Table 4).

## DISCUSSION

The obtained results revealed that the exposure of Bel-W3 tobacco plants to the ambient ozone resulted in intensified salicylic acid biosynthesis in leaves showing characteristic ozone injuries. This observation was previously reported by other authors for *Arabidopsis* genotype Cvi-0 – hyperaccumulating salicylic acid upon ozone exposure and developing severe HR-like lesions [39]. Other studies involving hybrid poplar clones with diverse sensitivity to ozone confirmed the involvement of salicylic acid in influencing cell death [24]. In our experiment, salicylic acid content in leaves of

tobacco Bel-W3 showing 100% injuries amounted to ~20 μg $g^{-1}$ FW. This was markedly higher than the values obtained by other researchers in leaves of a wild tobacco following 6-hour treatment with ozone at the concentration of 200 ppb in fumigation chambers [33].

During the first phase of investigations (one-site exposure/multi-leaf analysis), the exposure of the Bel-W3 plants to ambient air caused a fourfold increase in the content of free salicylic acid and a nearly twentyfold increase in the total salicylic acid in leaves with visible ozone-caused injuries (compared with uninjured control plants). In the ozone-tolerant Bel-B cultivar the exposure led only to a slight increase in the salicylic acid content, thus confirming earlier experiments conducted in controlled conditions [35]. High concentrations of salicylic acid glucoside showing no direct biological activity confirmed the existence of a mechanism for the storage of a locally and systemically active acidic form, probably for the purpose of further exposure and cross-tolerance [46], and/or effective detoxification of salicylic acid, whose local concentration may exceed the phytotoxicity threshold [17, 25].

The exposure of the Bel-W3 tobacco plants caused a decline in the ratio between the free and bound salicylic acid (the percentage of free salicylic acid in the total content), from about 30% before the exposure to about 12% after it, which can indicate a regulatory function of salicylic acid in the biosynthesis of its glucoside, probably following the activation of a glucosyltransferase catalysing its conjugation with glucose [17].

As evident from earlier investigations on the biosynthesis of salicylic acid, benzoic acid, alongside coumaric acid, is recognized as a precursor of this compound in tobacco leaves inoculated with TMV [43], whereas the hydroxylation reaction of the benzoic acid on the ortho position is catalysed by benzoic acid 2-hydroxylase [26]. Our results indicate that, also in the case of the ozone stress, an increase in the BA2H activity occurs and remains in a strict correlation with the total content of salicylic acid. Hence, it can be stated that in conditions of ozone stress, benzoic acid is a precursor of salicylic acid. The observed values of BA2H activity in leaves of the Bel-W3 cultivar were about ten percent of the highest levels recorded during the infection with TMV [26], but they differed significantly from the activity of this enzyme in Bel-W3 plants not exposed to ozone. A slightly weaker correlation of the BA2H activity with the content of free salicylic acid indicates probably a rapid transformation of this compound into the glucoside followed by free SA release.

It can be also assumed that the enhanced biosynthesis of salicylic acid in leaves of the Bel-W3 plants confirms its involvement in PCD via the impact on the hydrogen peroxide metabolism in response to ozone. According to Durner et al. [8], salicylic acid influences the cellular redox state and potentiates ROS generation followed by a cell death and formation of ozone-caused lesion. In contrary, plants compromised for salicylic acid accumulation or having reduced the ability to perceive SA (NahG plants and hybrid poplar NE-388 clone, respectively) exhibit only week induction of antioxidant system and the lack of ozone-induced PCD [24, 44]. Our results supplement earlier studies on different ROS generation (primarily $H_2O_2$) in leaves of both tobacco cultivars exposed to ozone in fumigation chambers [38, 47, 54]. These studies

*Table 5*

Mean values of air pollutants concentration and meteorological parameters at exposure site located in Botanical Garden during exposure series in year 2006

| Exposure series (2006) | Air contamination | | | | | | Meteorological parameters | | |
|---|---|---|---|---|---|---|---|---|---|
| | $O_3$ | AOT 40 | CO | $NO_x$ | $C_6H_6$ | PM | TP | RAD | WS |
| | [µg m$^{-3}$] | [(µg m$^{-3}$) h] | [mg g$^{-3}$] | [µg m$^{-3}$] | [µg m$^{-3}$] | [µg m$^{-3}$] | [°C] | [W m$^{-2}$] | [m s$^{-1}$] |
| 29.05–11.06 | 55.7 | 837.3 | 0.28 | 31.6 | 0 | 11.9 | 11.4 | 230 | 1.4 |
| 12.06–25.06 | 80.2 | 4755.6 | 0.30 | 25.8 | 0 | 20.8 | 20.3 | 256 | 0.9 |
| 26.06–09.07 | 87.0 | 5100.8 | 0.21 | 20.5 | 0 | 18.1 | 22.1 | 296 | 1.1 |
| 24.07–06.08 | 71.2 | 2878.7 | 0.19 | 24.1 | 0.17 | 18.6 | 20.7 | 190 | 1.2 |
| 07.08–20.08 | 51.7 | 1098.5 | 0.28 | 27.6 | 0.33 | 16.9 | 17.2 | 165 | 1.2 |
| 21.08–04.09 | 41.6 | 103.3 | 0.28 | 28.3 | 0.31 | 14.4 | 14.6 | 123 | 1.6 |
| Mean | 64.6 | 2462.37 | 0.26 | 26.3 | 0.14 | 16.8 | 17.7 | 210 | 1.2 |

Data from automatic monitoring conducted by WIOS (AOT 40 – accumulated ozone exposure over a threshold of 40 ppb, PM – particulate matter, TP – ambient temperature, RAD – solar radiation, WS – wind speed).

corroborated the biphasic generation of ROS, although in the case of the Bel-B cultivar only the first phase in the course of exposure was observed as a result of ozone reactions in the apoplast. In case of the Bel-W3 cultivar, also the second phase occurred after the exposure, resulted from the induction of defense pathways and a controlled intracellular response to ozone, including biosynthesis of salicylic acid.

During the entire course of investigations ozone-induced injuries were observed only on leaves of the Bel-W3 cultivar which indicates that in Poznan and its surroundings the threat to plants posed by tropospheric ozone was moderate [27, 28]. The mean value of injury during the first two years of studies (2004/2005) only slightly exceeded 4%. Simultaneously, average ozone concentration in Poland was low and comparable to other central and northern European countries. Only during the third year of experiments (2006) did the collected plant material exhibit full-scale injuries ranging from 0 to 100% with the average nearly 18%. According to the EEA Technical Report [9] the ozone concentrations during summer months in 2006 were much higher at whole Europe area (including northern countries). Average ozone concentration measured at the exposure site located in Botanical Garden reached almost 65 $\mu g\ m^{-3}$ (AOT 40 = 5101 $\mu g\ m^{-3}$ h) (Table 5). The highest average ozone concentration in air was noticed for the exposure series from the 26th of July till the 9th of September together with the highest air temperature, solar radiation, and the lowest concentration of $NO_x$ (Table 5). Low values of the last one are strongly connected with its consumption during ozone creation process.

In 2006, the lowest injuries of tobacco Bel-W3 leaves were observed in the city centre (Kaponiera Round), and the highest in the suburban and rural areas (Botanical Garden and Tarnowo Podgorne, respectively). This phenomenon was observed in many European cities as the effect of transport of ozone precursors far away from their emission sources and creation of ozone in favorable meteorological conditions at remote areas [14, 22, 23]. Moreover, high leaf injury of tobacco plants in the suburban area and low in the city centre may be connected with ozone reaction with nitrogen oxides, which show higher levels in city centers due to more intense car traffic [5].

The value of injuries depended significantly on the leaf age. The oldest as well as the centrally located leaves suffered the most severe injuries and the developed necroses covered the leaf blade surfaces uniformly. The youngest leaves, on the other hand, suffered the least damage and slight necroses were observed only on their peaks. According to authors of earlier publications dealing with the impact of a single exposure of the tobacco Bel-W3 plants to ozone in controlled conditions, the first injuries appeared, primarily, on central leaves. Rare damage to younger and older leaves appeared gradually, at the leaf peak or base respectively [12, 47, 49]. The above researchers maintained that such distributions of necroses could be attributed to the degree of development of stomata and the intensity of the gas exchange. Simultaneously, the highest content of both free and bound salicylic acid and the highest BA2H activity were observed for leaf number 6, 7 and 8 of the Bel-W3 plants, while in the case of the Bel-B cultivar, which failed to exhibit ozone-induced injuries, the content of salicylic acid in consecutive leaves remained quite constant, indicating

a constant concentration of this compound in conditions of homeostasis. The impact of leaf age on the intensity of its response to ozone was confirmed by presenting the content of salicylic acid and BA2H activity falling to one percent of the observed injuries. The youngest leaves, having been exposed for the shortest time (i.e. leaf 9), responded most strongly to the presence of ozone due to enhanced salicylic acid biosynthesis during the early phases of lesion development. This corroborated the involvement of this compound in the phases of initiation and propagation of ozone-induced necroses proposed by other authors. According to Pasqualini et al. [35] and Rao et al. [40], salicylic acid plays a central role in induction of the oxidative burst as well as in intercellular signal transduction, informing adjacent cells about stress factor, thus propagating the ozone-caused lesions.

In the case of investigations carried out in ambient conditions and a two-week exposure, it was necessary to select a representative leaf in order to obtain an objective assessment of the ozone impact on plants. On the basis of investigations conducted during the first two years of experiment, we selected leaf number 7 counted from the plant base, because in the majority of cases, following the two-week plant exposure, this leaf was fully developed and did not exhibit symptoms of aging. In addition, at high injury values, lower situated leaves, i.e. 5th and 6th, were frequently dry, and this made it impossible to carry out a comparative analysis on exposure series with less intense ozone symptoms. Simultaneously, at low injuries of leaves 5 and 6, ozone-induced necrosis was most frequently observed only as far as leaf 7.

In the last year of investigations we carried out a biomonitoring experiment in which the levels of salicylic acid and ozone-induced injuries were assessed for leaf 7 of the Bel-W3 cultivar and assumed as the most representative. The performed statistical analysis allowed us to confirm almost linear correlation between the content of free and bound salicylic acid and the observed injuries. Moreover, it is worth to identify if salicylic acid biosynthesis precedes displaying of injuries of the Bel-W3 leaves in order to employ this cultivar as a short-time exposure bioindicator of troposheric ozone, and to assess the ozone impact on yet uninjured leaves.

## CONCLUSIONS

Exposure of Bel-W3 tobacco plants to ambient ozone leads to the biosynthesis of salicylic acid, which accompanies the development of characteristic injuries on leaf-blades. At the same time, exposure of the Bel-B cultivar fails to cause either the appearance of ozone-induced necroses or a significant increase in the content of salicylic acid, pointing to the involvement of this compound in the development of injuries. Salicylic acid occurs in tobacco leaves both in the free as well as glucoside forms, but in the case of ozone-sensitive cultivar exposure to tropospheric ozone causes changes in the quantitative ratio of these two forms in favour of the salicylic acid glucoside. The development of ozone-induced injuries is accompanied by an increased activity of benzoic acid 2-hydroxylase strongly correlated with salicylic acid glucoside content, confirming the involvement of this enzyme in the biosynthesis

of salicylic acid in conditions of ozone stress and, probably, offering evidence for a rapid transformation of the acid form into a conjugate with glucose followed by the release of an active form. Both the development of ozone-caused injuries and salicylic acid biosynthesis depend on the developmental phase of tobacco plant leaves. Therefore, the selection of the leaf for bioindication studies should take into consideration the growth rate of plants in climatic conditions characteristic for the given region. In view of the close correlation of the free salicylic acid content with the injuries of the 7th leaf of the Bel-W3 cultivar, this compound can be used as a biomarker of the ozone stress and ozone-induced cell death in conditions of ambient air.

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