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Study of changes in the degree of tobacco leaf injury caused by tropospheric ozone

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SUMMARY

Air pollution by ground-level ozone in Poland has recently been increasing, and is connected with the increased number of cars, the main source of ozone precursors. The phytotoxic influence of this pollutant was discovered in the 1950s, when visible injuries to tobacco and grapevine leaves were observed. Bioindicative methods are very important for evaluating the air pollution level, as well as for assessment of the impact of air pollutants on living organisms. The tobacco plant and its ozone-sensitive Bel W3 cultivar are well known bioindicators. This cultivar was used in the studies presented here. Plants were exposed in Poznań city centre during the 2002–2006 growing seasons. A statistical model of multivariate analysis of variance is set up to determine the differences between selected years and leaves with respect to the degree of visible tobacco leaf injury. Statistical differences in the degree of leaf injury were observed between years, as well as variation among exposure series during growing seasons. These differences were connected with meteorological conditions, which are among the factors promoting tropospheric ozone creation. Statistical differences of leaf injuries were not observed between individual leaves.

Key words: multivariate analysis of variance, canonical variates, tropospheric ozone, bioindication

1. Introduction

The number of cars has been increasing in Poland systematically in the last few decades, reaching 12 million when last reported (*Statistical information and elaborations,* 2007). Together with industry, road traffic is the largest air pollution source. The main air pollutants emitted by cars are nitrogen oxides, carbon oxides and hydrocarbons, all ozone precursors whose concentration is incre-asing in Poland. Ground-level ozone is a secondary air pollutant created during photochemical reactions from the above-mentioned precursors. Hence the highest ozone concentrations are usually observed during hot summer days. The Intergovernmental Panel on Climate Change conducts investigations to predict selected air pollutants' concentration in the future. Tropospheric ozone is a greenhouse gas, and all scenarios predict a greater or lesser increase in its concentration up to the year 2100 (Climate Change, 2007).

Ground-level ozone has been adjudged the most important air pollutant. Its high concentration can cause crop losses and visible symptoms on trees, horticultural plants and vegetables, sometimes decreasing the economic value of plants (Heggestad, 1991; Meyer *et al.*, 2000).

The European Union ozone directive (Directive 2008/50/EC) determines limiting values of tropospheric ozone concentration with regard to plant and ecosystem protection. Investigations with bioindicators for tropospheric ozone have also been recommended. Bioindicators are very useful tools, because of their visible response to the accumulation of air pollutants. Ozone is a very reactive air pollutant that does not accumulate in plants. Hence bioindicators have revealed only visual symptoms that could be used for detection of this air pollutant. The tobacco plant and its two cultivars (Bel W3 and Bel B) have been recognized as sensitive and resistant to tropospheric ozone (Heggestad, 1991). There have been many biomonitoring projects where tobacco plants were used as ozone bioindicators (Calatayud *et al.*, 2007; Cuny *et al.*, 2004; Godzik, 1998a; Klumpp *et al.*, 2006b; Saitanis and Karandinos, 2001). The ozone-sensitive tobacco cultivar exhibits a visual response to ground-level ozone in the form of bifacial necrosis, while the resistant one shows no evident symptoms. This feature is very useful in the practice of biomonitoring – by placing both cultivars together we can be sure that any injuries to Bel W3 are caused by ozone. Tobacco plants are the best known tropospheric ozone bioindicators (Sant'Anna *et al.*, 2008). Previous investigations have revealed that the degree of leaf injury is correlated with tropospheric ozone concentration (Borowiak, 2005). Hence we are able to use this bioindicator to evaluate the tropospheric ozone level.

There have been only a few experiments with ozone bioindicators in Poland, most of them conducted in the southern part of the country, while the rest of Poland was believed to have rather low ozone concentrations. However, automatic monitoring conducted in the Poznań city area revealed ozone concentrations that might negatively influence plants. Hence the present investigations conducted with bioindicators are necessary to obtain information about the usefulness of the tobacco plant as a bioindicator in the Poznan city area. Moreover, these investigations could help to ascertain which leaf is the best for further biochemical investigations of the plant's internal response to ozone. A statistical model of multivariate analysis of variance was specially employed to indicate the differences between individual years and leaves in degree of visible tobacco leaf injury.

2. Materials and methods

2.1. Experimental design

Tobacco plants were used in the present studies as bioindicators of tropospheric ozone. Two cultivars with different ozone sensitivity were chosen. The ozone-resistant (Bel B) cultivar, which exhibits no visual symptoms, was treated as the control with respect to the sensitive one (Bel W3). The latter cultivar revealed bifacial necrosis as a response of the plant to ozone. Plants were located at the exposure site in Poznań city centre (Central-Western Poland), where automatic monitoring of air pollution is carried out by the Provincial Environmental Inspectorate of Wielkopolska. This made possible a comparison of the results of biomonitoring with those from real tropospheric ozone monitoring.

Cultivation and exposure were established according to a standardized German method (Klumpp *et al.*, 2006a). Plants were cultivated under green-house conditions for eight weeks, and afterwards transported for two weeks of exposure in the city centre. Five plants of the sensitive cultivar and one of the resistant one were located at the exposure site. During one vegetative season plants were exposed in 6 series, each of two weeks' duration, from the beginning of June until the beginning of September, in the years 2002 to 2006 (Table 1).

Table 1. Dates of tobacco plant exposure series in the 2002–2006 growing seasons

	Exposure dates in growing seasons				
Exposure series number	2002	2003	2004	2005	2006
1	10/06–23/06	09/06–22/06	14/06–27/06	14/06–27/06	12/06–25/06
2	24/06–07/07	23/06–06/07	28/06–11/07	28/06–11/07	26/06–09/06
3	08/07–21/07	07/07–20/07	12/07–25/07	12/07–25/07	10/07–23/07
4	22/07–04/08	21/07–03/08	26/07–08/08	26/07–08/08	24/07–06/08
5	05/08–25/08	04/08–17/08	09/08–22/08	09/08–22/08	07/08–20/08
6	26/08–08/09	18/08–31/08	23/08–05/09	23/08–05/09	21/08–03/09

The visible leaf injuries of the sensitive cultivar were measured after each exposure period. The symptoms were evaluated on the 4^{th}, 5^{th} and 6^{th} leaf, counted from the bottom, as a percentage of injured leaf area, and were presented on a scale of 0–1.

2.2. Statistical model

Multivariate analysis of variance in double classification was used for interpretation of the data obtained. Let $\mathbf{y}_{kj}$ denote the 6×1 vector of mean values of leaf injury degrees coming from the k-th year (k = 1,..., 5) and j-th leaf (j = 4, 5, 6), and let $N = \sum_k \sum_j 1$. The multivariate linear model (Caliński *et al.* 1987, Seber 1984) can be written in the form:

$$\mathbf{Y} = \mathbf{1}_N \boldsymbol{\mu}' + \mathbf{X}_1 \boldsymbol{\alpha} + \mathbf{X}_2 \boldsymbol{\beta} + \mathbf{e} \tag{1}$$

where $\mathbf{Y} = [\mathbf{y}_{1,4}, ..., \mathbf{y}_{5,6}]'$ is the N×6 matrix of observations, $\boldsymbol{\mu}$ is the 6×1 vector of general means, $\boldsymbol{\alpha}$ is the 5×6 matrix of year parameters, $\boldsymbol{\beta}$ is the 3×6 matrix of leaf parameters, $\mathbf{X}_1$, $\mathbf{X}_2$ are design matrices, and $\mathbf{e}$ is the N×1 matrix of errors.

Let us consider the hypothesis $\mathrm{H}_{01}: \mathbf{C}_1\boldsymbol{\alpha} = \mathbf{0}$, where $\mathbf{C}_1$ is, for example, the 5 × 5 matrix as follows: $\mathbf{C}_1 = \mathbf{I}_5 - \frac{1}{5}\mathbf{1}_5\mathbf{1}'_5$. The hypothesis H_{01} was tested according to Wilks's criterion (Morrison 1967, Seber 1980). Hypothesis H_{01} asserts the absence of differences in leaf injury degree in years in comparison with the mean of the parameters of all years. After rejection of H_{01}, we determine which elements of the $\boldsymbol{\alpha}$ matrix are responsible for it. Tests of hypotheses $\mathrm{H}_{01,k}: \mathbf{c}'_{1k}\boldsymbol{\alpha} = \mathbf{0}$, ($\mathbf{c}'_{1k}$ being the k-th row of matrix $\mathbf{C}_1$) make it possible to identify years differing from the average year. The tests $\mathrm{H}_{01,ki}: \mathbf{c}'_{1k}\boldsymbol{\alpha}\mathbf{m}_i = 0$ ($\mathbf{m}_i$ being the i-th column of matrix $\mathbf{I}_5$) allow us to determine which elements of the matrix $\mathbf{c}'_{1k}\boldsymbol{\alpha} = \mathbf{0}$ caused the rejection of hypothesis $\mathrm{H}_{01,k}$ (Lejeune and Caliński, 2000).

Let us consider the hypothesis $\mathrm{H}_{02}: \mathbf{C}_2\boldsymbol{\beta} = \mathbf{0}$, where $\mathbf{C}_2$ is for example the 3 × 3 matrix as follows: $\mathbf{C}_2 = \mathbf{I}_3 - \frac{1}{3}\mathbf{1}_3\mathbf{1}'_3$. The hypothesis H_{02} was tested according to Wilks's criterion (Anderson, 2003, Morrison, 1967, Seber, 1980). Hypothesis H_{02} asserts the absence of differences in leaf injury degree in an individual leaf in comparison with the mean of the parameters of all individual leaves.

The results of multivariate analysis of variance were presented graphically in the space of the first two canonical variates (Lejeune and Caliński, 2000, Kayzer *et al.*, 2009).

3. Results and discussion

The ozone concentration during all five years was at a level causing visible leaf injuries only to the sensitive cultivar. Tropospheric ozone concentration is presented as an AOT 40 value for plants' and ecosystems' responses. This is an accumulated value over the threshold 40 ppb over the period 8.00 to 20.00. The investigations revealed a positive linear correlation between ozone and visible leaf injury (Figure 1). Hence we could confirm the usefulness of tobacco plants as a bioindicator of tropospheric ozone in the Poznań city area.

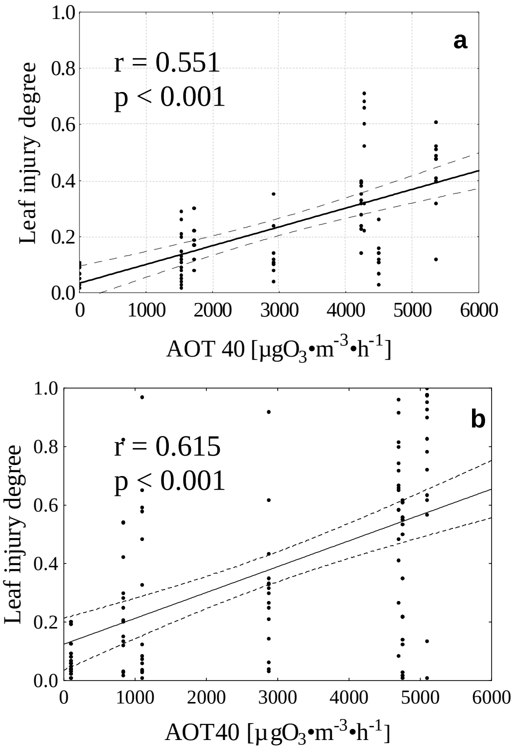

Figure 1. Leaf injury degree of ozone-sensitive tobacco cultivar and ozone concentration during experimental years 2002 (a) and 2006 (b)

The hypothesis H_{01} was rejected at the given significance level, because the test statistic value $F = 5.93$ is larger than the critical value $F_{24;6,4} = 3.84$ $(p = 0.017)$. Comparing the computed value $F = 1.05$ to the critical value $F_{12;1} = 243.9$, we can observe that there was insufficient evidence to reject H_{02} at the level $\alpha = 0.05$ $(p = 0.65)$. The analysis revealed that leaf injury degree is dependent on the year and partially on the leaf number.

The highest values of visible leaf injury were observed in 2002 during the t_1 and t_2 exposure series, as well as in 2005 in the t_3, t_4 and t_6 exposure series (Table 2, Figure 2). The lowest values of visible leaf symptoms were found in 2003 and 2004 in the t_2, t_3, and t_6 exposure series, and in the first term of exposure in 2004 and 2006. Levels of leaf injuries of sensitive tobacco plants in Poznan city in 2002 were comparable to symptoms observed at the same time in Düsseldorf and Edinburgh (Klump *et al.*, 2004). Variability of ozone-caused tobacco injuries during the growing season was also observed in studies conducted in the southern part of Poland (in Kraków province). During these investigations a high level of leaf injury was observed in June and remained at a medium level until the end of August (Godzik, 1998b). High values of ozone-caused injuries of tobacco leaves in June and July were also noted in Polish mountain National Parks – again in the southern part of Poland (Godzik, 2000). Analogous results were obtained in bioindication studies in Italy, Greece and the Swiss mountains (Novak *et al.*, 2003; Saitanis, 2003; Toncelli and Lorenzini, 1999).

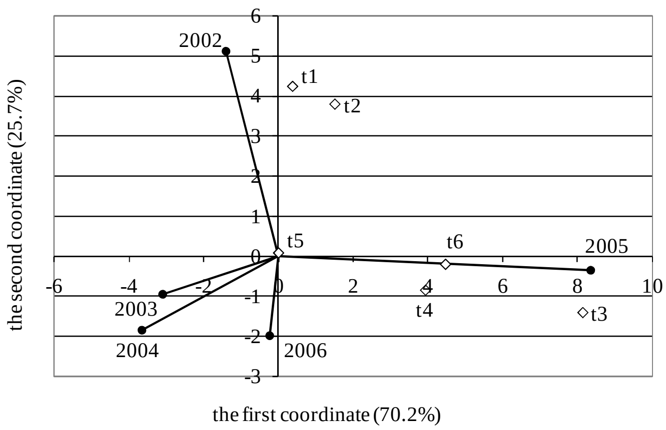

Figure 2. Position of selected years relative to the average year in the space of the first two canonical variates and distribution of the particular terms of expositions in the dual space of canonical variates (values of dual canonical coordinates are multiplied by 10)

Table 2. Evaluations of differences in the degree of leaf injury of tobacco plants in selected years in comparison to the average year **($C_1\alpha$)**

Year	$C_1\alpha$					
	T_1	t_2	t_3	t_4	t_5	t_6
2002	0.202**	0.207*	-0.113	-0.068	0.004	-0.052
2003	-0.001	-0.163	-0.219*	-0.097	-0.006	-0.072*
2004	-0.105*	-0.164	-0.222*	-0.071	0.011	-0.063*
2005	0.011	0.007	0.356**	0.188**	0.003	0.237**
2006	-0.107*	0.114	0.197*	0.049	-0.011	-0.049

* significant at level $\alpha = 0.05$
** significant at level $\alpha = 0.01$

The results obtained with the model of multivariate analysis of variance confirmed data obtained from air pollution and meteorological monitoring. Tropospheric ozone concentration is a result of a combination of several factors, such as ozone precursor concentrations (e.g. nitrogen oxides) and favourable meteorological conditions (such as solar radiation and temperature). In 2004 low temperatures and solar radiation resulted in low ozone concentration, while in 2002 and 2006 we recorded higher temperatures and solar radiation (especially for 2006). There are no ozone and solar radiation data for 2003, but on the basis of temperature level, we could assume a low ozone concentration during that growing season. The bioindication research confirmed these assumptions. The highest ozone concentration was observed in 2002 and 2006 – similar to the indicated results of multivariate analysis of variance of visible leaf tobacco injury (Table 3). Hence the method could be a useful tool for further data presentation of multiyear air biomonitoring results.

In spite of the lack of significant differences between injuries of individual leaves, it is possible to indicate the greatest symptoms at the fourth and the least at the sixth leaf, counting from the bottom (Table 4, Figure 3). Based on our results we can select the proper leaf for further investigation of internal plant response to ozone. Thus the results suggest that the fifth leaf is the best, because of its maturity before exposure. The younger sixth leaf was developing during exposure, and ozone did not affect the whole leaf area from the beginning of exposure. We could assume that in further bioindication research with tobacco plants, using the same experimental schedule, the fifth leaf counted from the bottom might be used as an indicator of internal plant response to ozone.

Table 3. Selected meteorological parameters (temperature and solar radiation) and air pollutants (ozone and nitrogen dioxide/nitrogen oxides) in experimental series and years

Exposure series	Temperature [ºC]	Total solar radiation [W·m^{-2}]	AOT40 [μg·m^{-3}·h^{-1}]	Nitrogen dioxide [μg·m^{-3}]
		2002		
1	20.7	215	4269.9	28
2	18.3	187	4226.2	26
3	22.6	189	5345.5	22
4	21.6	166	2922.0	27
5	21.5	150	1524.1	25
6	22.9	188	4488.4	39
		2003		
1	19.1	x	x	29
2	18.8	x	x	28
3	20.2	x	x	27
4	23.0	x	x	34
5	20.7	x	x	31
6	17.9	x	x	38
		2004		
1	16.6	186	140.1	19
2	17.6	205	230.2	23
3	19.3	164	781.3	24
4	21.6	196	649.7	22
5	22.0	191	991.2	25
6	17.7	153	232.0	28
		2005		
1	18.9	289	1545.0	31*
2	19.0	252	1006	25
3	18.3	205	119.5	33
4	18.7	199	290.0	34
5	16.4	188	323.5	31
6	17.4	218	1079.0	67
		2006		
1	20.7	256	4755.6	22*
2	22.4	296	5100.8	17
3	23.1	262	4689.6	23
4	21.1	190	2878.7	22
5	17.6	166	1098.5	28
6	15.0	124	103.3	28

*in 2005 and 2006 nitrogen oxides were measured

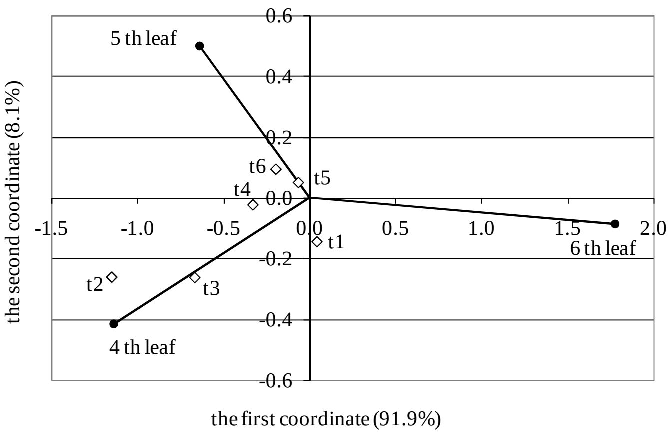

Figure 3. Position of particular leaves relative to the average exposure series in the space of the first two canonical variates and distribution of the particular terms of expositions in the dual space of canonical variates (values of dual canonical coordinates are multiplied by 3)

Table 4. Evaluations of differences in the leaf injury degree of tobacco plants in selected exposure series in comparison with the average level of damage to an individual leaf **($C_2\beta$)**

Individual leaf number	$C_2\beta$					
	t_1	t_2	t_3	t_4	t_5	t_6
4th leaf	0.010	0.113	0.076	0.027	0.000	0.006
5th leaf	-0.018	0.020	-0.001	0.012	0.009	0.019
6th leaf	0.008	-0.133	-0.075	-0.039	-0.009	-0.025

4. Conclusions

The statistical model of multivariate analysis of variance performed specially for the purposes of these data is a good tool for verification of differences between degrees of tobacco leaf injury caused by tropospheric ozone. On the basis of this analysis the following results were obtained:

1. Differences in visible ozone-caused leaf injuries between years, as well as variations between exposure series in different growing seasons, were

found. These differences were connected with meteorological conditions, one of the factors affecting tropospheric ozone creation.

2. No significant differences were observed between individual leaves, but for further studies of internal plant response to tropospheric ozone the 5th leaf might be chosen because of its moderate ozone symptoms and maturity before exposure.

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