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Response of an ozone indicator plant before and after installation of a desulphurization device at a thermal power plant

Odziv bioindikatorskih rastlin za ozon pred in po namestitvi odžveplevalne naprave na termoenergetskem objektu

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Abstract: The main goal of the research was to compare the plant response to air pollutants in the rural environment (Zavodnje village) before and after the installation of a desulphurisation device at unit 5 of the Šoštanj Thermal Power Plant (Slovenia). The installation of the cleaning device for SO_2 in the year 2001 caused very significant reduction of annual average SO_2 emission and immission, while concentrations of dust particles, O_3, NO_x, and CO_2 in Zavodnje remained unchanged. On the other hand the average concentrations of ozone during seasonal experimental period increased after 2001, but there were no significant differences in AOT40. The impact of O_3 in combination with other air pollutants was studied at Zavodnje in the period 1996–2003 using white clover (*Trifolium repens* 'Regal') on the basis of visible ozone injuries of leaves, biomass reduction and by analysis of chosen biochemical stress parameters in leaves – the content of plant pigments and antioxidants. The effects of reduction of SO_2 after 2001 were observed in the biochemical responses of white clover plants, which showed better vitality. Despite of that, there was no significant difference in the level of plant injury by ozone between the period 1996–2000 and period 2001–2003. Biomass ratio CN-S/CN-R was also unaffected.

Key words: air pollution, desulphurization devices, *Trifolium repens* 'Regal'; ozone, sulphur dioxide, plant pigments and antioxidants, ozone injuries, biomass

Izvleček: Glavni namen raziskave je bil primerjava odziva rastlin na zračna onesnažila v ruralnem okolju (vas Zavodnje) pred in po namestitvi odžveplevalne naprave na 5. bloku Termoelektrarne Šoštanj. Namestitev te naprave v letu 2001 je povzročila značilno zmanjšanje letnih povprečnih imisij in emisij SO_2, medtem ko se koncentracije prašnih delcev, O_3, NO_x, and CO_2 v Zavodnjah niso spremenile. Na poskusnem mestu, blizu ANAS postaje v Zavodnjah, smo v obdobju 1996–2003 spremljali vpliv O_3 v kombinaciji z drugimi zračnimi onesnažili na deteljo (*Trifolium repens* 'Regal') preko ocenjevanja vidnih ozonskih poškodb na listih detelje, sprememb v biomasi in analizah rastlinskih barvil in antioksidantov v listih. Učinek zmanjšanja koncentracije SO_2 v okolju po letu 2001 se je na rastlinah pokazal na biokemijskem nivoju, medtem ko ni bilo učinka na zmanjšanje poškodb na listih ter biomasi.

Ključne besede: onesnaženost zraka, odžveplevalne naprave, *Trifolium repens* 'Regal'; ozon, žveplov dioksid, rastlinska barvila in antioksidanti, ozonske poškodbe, biomasa

Introduction

Increasing emission of air pollutants (NO_x, VOCs) from traffic, thermal power plants (NO_x, SO_2) and transboundary transport are the main source of photo-oxidants. Sulphur dioxide (SO_2) pollution in Slovenia was limited to larger towns and valleys around industrial and power plants (the Zasavje region, Mežica valley and Šalek basin). After the year 2001, when the new desulphurisation device on the Šoštanj thermal power plant (ŠTPP) (Šalek basin) was installed, over 95% of SO_2 was removed (Vrtačnik and Ribarič-Lasnik 2001). Other pollutants, especially nitrogen oxides (NO_x) and hydrocarbons (VOCs), remained a problem and became increasingly problematic with increasing traffic. Nitrogen oxides and hydrocarbons participate in many photochemical reactions in the atmosphere, the results of which are photo-oxidant ozone (O_3) and a number of organic compounds. O_3 travels with air masses, rarely reacting at the place of its formation, but more often far away (Krupa and Manning 1988). The most exposed places are rural areas, where the main plant production takes place. Pollution of air with O_3 occurs in summer, during the main growing period. As a strong oxidant, O_3 is highly reactive with various forms of organic molecules in living systems and is extremely harmful to organisms.

Different air pollutants can act synergistically or antagonistically in the environment. The adverse effects of O_3 and SO_2 on organisms are more pronounced if they act together than if they act separately (Mehlhorn et al. 1986, Lepper 1992). The direct damaging effect of ozone is due to the generation of free radicals (Peñarrubia and Moreno 1999, Iriti and Faoro 2008). Plants respond with a defence mechanism against free radicals, based on the ability of specific enzymes and the activity of antioxidants to prevent, reduce or stop chain oxidations (Perl-Treves and Perl 2002, Jones 2006, Halliwell and Gutteridge 2007). Ozone is unlikely to reach the chloroplast, but it nevertheless causes pigment bleaching and lipid peroxidation (Harbinson and Hedley 1993). On the other hand, the phytotoxic effect of sulphur dioxide consists mainly of acidification of cell compartments and formation of free radicals during oxidation of sulphite to sulphate (Rennenberg and Polle 1994). Additional effect is inhibition of Rubisco, the major enzyme of carbon dioxide fixation (Malhotra and Khan 1985, Okpodu et al. 1996, Lee et al. 1997).

To defend against both pollutants, O_3 and SO_2, a strong constitutive antioxidative defence is required. The influence of air pollutants on organisms depends on many factors, such as climate, nutrients, predisposition and age (Krupa and Manning 1988). The use of bioindicators is an effective method for determining the effects of air pollutants on organisms under given environmental conditions and for specifying limiting and critical values for pollutant concentrations.

Numerous reports have documented the effects of mixtures of O_3 and SO_2 on visible injury and plant growth and yield. These effects have shown responses ranging from antagonistic to synergistic (Guderian 1985). O_3 impacts often manifest themselves as reduced plant growth and altered carbohydrate allocation patterns at the level of the whole plant (Pell et al. 1994, Grantz 2003). Reduced partitioning of photosynthates from leaves to sink tissues such as roots is commonly observed (Barnes et al. 1998). These impacts of O_3 on allocation have profound effects on root function, plant water relations and integration of whole plant function (Grantz 2003). In contrast to ozone, SO_2 can act principally as a nutrient, as sulphur-containing compound are required by the plants. Absorbed SO_2 is used to synthesize glutathione, which is translocated in the phloem to the roots (Peñarrubia and Moreno 1999). Flagler and Youngner (1982) found that SO_2 affected tall fescue, but not nearly as severely as O_3. Number of tillers was significantly reduced by an interaction of the two pollutants. Neither O_3 nor SO_2 had an effect singly, but at the high O_3 level, the addition of SO_2 caused an 18% reduction in number of tillers.

The main goal of this research was to compare the response of two clones of white clover NC-S (less O_3 tolerant) and NC-R (more O_3 tolerant) to air pollutants, before and after the reduction of SO_2 pollution from the ŠTPP in the rural environment. We expected that after reducing SO_2 emission, the synergistic effect of SO_2 and O_3 would be diminished; reducing the harmful effect on plants.

Materials and methods

Study area

The experiment took place from 1996 to 2003 in Zavodnje near Šoštanj (734 m above sea level, 46°25'37.51'', 14°59'39.14'') near an automatic measurement station of the ecological information centre of the Šoštanj thermal power plant (ŠTPP), where SO_2, NO_x, NO_2 and O_3 immission concentrations, air temperature, relative air humidity, wind speed and direction are measured every 30 minutes. Zavodnje was chosen as a test site, because it is the only site within the area of influence of the ŠTPP that is situated in a rural environment, where the concentrations of O_3 and its precursors have been measured for many years.

AOT40 – ozone biological impact

AOT40, cumulative indices of ozone exposure in the period of three months (May to July) were calculated to evaluate the biological impact, which represent the sum of the differences between the hourly O_3 concentrations (in ppb) and a threshold value of 40 ppb, for each hour in the interval 08:00-20:00h (Directive 2002/3/EC in Official Journal of European Communities, L. 67, March 9, 2002).

Plant material and method of exposure

Two clones of white clover (*Trifolium repens* 'Regal'), variety NC-S (O_3 sensitive clone) and NC-R (O_3 tolerant clone) were chosen from the group of plants known to be indicators of ozone impact (Heagle et al. 1995). The plants were exposed to outdoor environmental conditions in the study area in Zavodnje. Every plant, which showed advanced symptoms of disease *(Polythrincium trifolii* Kunze*, Uromyces flectens* Lagerh.) and virus infection, was excluded from experiment. For control of aphids, corn earworm and armyworms, Japanese beetles and mites, plants were treated by bifenthrin (Talstar) or natural insecticide pyrethrin. For slug controls slug pellets (metaldehyde) were used. For fungal pathogen no fungicides were used, because it is believed that some of them (benomyl and triadimefon) offer some protection against ozone toxicity. Data of the specific exposure periods and harvests are shown in Tab. 1. One plant cutting of each white clover clone was planted in 12 L pot in greenhouse and after rooting period placed in a field for whole experimental season. New plants were used each year, provided by the ICP-Vegetation coordinator, Bangor, UK. Pots had self-irrigation system. There were 20 replications of each clone (NC-S, O_3 sensitive clone; NC-R, O_3 tolerant clone) every year. The experiment was carried out according to the protocol of the ICP Vegetation programme (Hayes et al. 2006).

Determination of ozone effects

The extent of visible ozone injury (white dots on upper surface of clover leaflets) was assessed weekly by calculating the percentage of injured leaves in each pot. All leaves, which were completely expanded, were used for assessing the injury class, based on a 0 to 6 scale (0: no injures, 1: very slight injury, occurrence of the first symptoms, 2: slight injury, 1-5% of the leaves with slight injury, 3: moderate injury, 5-25% of the leaves with injury, 4: heavy injury, 25-50% of the leaves injured, 5: very heavy injured, 50-90% of the leaves injured, 6: total injury, 90–100% of leaves are injured).

Clover biomass of each clone was determined at each harvest as dry weight of the above ground plant parts. Harvests were made every 28 days+/- 2 days (Tab. 1). The cutting height was 7 cm from the soil. The plant material was then dried to constant weight in an oven at 80 °C. The yield loss due to ozone impact was calculated by the ratio of biomass of the two clones, NC-S/NC-R. The yield loss was then calculated on the pot surface area to give the yield loss per hectare.

Plant material (leaves, which were completely expanded) for the analysis of plant pigments (chlorophylls a and b, zeaxanthine and lutein) and antioxidants (ascorbic acid) were taken before 9 a.m. (Tab. 1). It was frozen in liquid nitrogen in the field and stored in a cool box during transport to the ERICo laboratory. Only leaf blades were used in biochemical analyses. Ascorbic acid and plant pigments were analysed according to Pfeifhofer (1989) and Tausz (1996). The pigment and antioxidant analyses were made using high-pressure liquid chromatography (HPLC).

Table 1: Experimental season and dates of sampling of plants for biochemical analysis and measurement of biomass in different years.
Tabela 1: Poskusna obdobja in datumi vzorčenja rastlin za biokemijske analize in meritve biomase v različnih letih.

Experimental season	Dates of samplings and harvest	
31.5.1996 – 4.9.1996	1st harvest	11.7.1996
	2nd harvest	7.8.1996
	3rd harvest	4.9.1996
20.5.1997 – 8.8.1997	1st harvest	12.6.1997
	2nd harvest	11.7.1997
	3rd harvest	8.8.1997
20.5.1999 – 14.8.1999	1st harvest	20.6.1999
	2nd harvest	16.7.1999
	3rd harvest	14.8.1999
24.5.2000 – 17.8.2000	1st harvest	20.6.2000
	2nd harvest	19.7.2000
	3rd harvest	17.8.2000
19.6.2001 – 10.9.2001	1st harvest	18.7.2001
	2nd harvest	10.8.2001
	3rd harvest	10.9.2001
20.6.2002 – 18.9.2002	1st harvest	23.7.2002
	2nd harvest	23.8.2002
	3rd harvest	18.9.2002
13.5.2003 – 8.8.2003	1st harvest	16.6.2003
	2nd harvest	9.7.2003
	3rd harvest	8.8.2003

0.5 g lyophilised and ground leaves was used for determining the amount of ascorbic acid. Extraction was carried out in 5 mL of 1.5% meta-phosphoric acid (HPLC grade), and hexadecyltrimethylammonium bromide was used as the ion-pairing reagent. Ascorbic acid was detected directly at 248 nm. For determination of pigments little $CaCO_3$ to neutralize the solution, and 5 ml of acetone (HPLC grade) were added to 1 g of ground white clover leaves. After centrifugation (10 min, 4000 circ/min), supernatant was filtered through 0,45 µm regenerated-cellulose-membrane filter into dark phial (Pfeifhofer 1989). Zeaxanthine and lutein were detected at 430 nm, chlorophyll *a* at 430 nm and chlorophyll *b* at 458 nm (Tausz 1996). For all analysis three replicates of samples were measured.

Statistical analysis

Statistic analysis made use of Statistic for Windows 5.5. Data analysis was based on parametric statistical methods (t-test, ANOVA/MANOVA and LSD test).

Results and Disscussion

The yearly emissions of air pollutants from the ŠTPP and average yearly immissions of air pollutants at Zavodnje are listed in Tab. 2, together with the production of energy in the power plant in the period before and after the installation of the desulphurization device at unit 5. After 2000, the amount of energy produced in the ŠTPP increased considerably while, at the same time, the amount of SO_2 emissions per unit of energy produced was substantially reduced. Yearly concentrations of SO_2 at Zavodnje as well as SO_2 immission concentrations per amount of energy produced have decreased considerably since the desulphurization devices started operating. Annual concentrations of O_3 and the amount of dust particulates at Zavodnje have not significantly changed in the periods under comparison. There was no change in the emissions of NO_x and CO_2. Average immission concentrations of pollutants at Zavodnje, given in Tab. 3, are calculated only for the period of experiments (experimental season)

each year. The average immission concentration of O_3 at the experimental place in Zavodnje was between 1996 and 2000 73 µg/m^3; concentrations then increased significantly to an average value of 91.7 µg/m^3 in the period of 2001- 2003. The results of AOT40 values, calculated for the period May-July, showed similar trend with higher values in the period after the installation of the desulphurization device at unit 5, but differences was not statistical significant. Latter can be explained by better climate conditions for the ozone formation, based on higher air temperatures, since there were no differences between concentrations of O_3 precursors (NO_x ($NO+NO_2$) and NO_2) in the periods before and after the desulphurisation device started operating (Tab. 3). Ambient ozone concentration in experimental site was above the levels defined as phytotoxic for vegetation by EU Directive 92/72/CEE. Critical levels of AOT40 for protection agricultural plants for three month (May – July) is 3 000 ppb (Sanders et al. 1995). Levels of AOT40 (May – July) (Tab. 3) were above critical values during all experiment years, with increasing potential, indicating a serious potential threat for crops at Zavodnje and surroundings.

The contents of chlorophylls in the leaves of both clones of white clover increased significantly after installation of the desulphurization device (Fig. 1a,b,c). The most obvious increase occurred in chlorophyll a (NC-R: from 1.86 mg/g DW before 2001 to 6.46 mg/g DW after 2001 and NC-S: from 1.77 mg/g DW before 2001 to 6.06 mg/g DW after 2001), what shows better vitality of plants after 2001. The processes involved in photosynthetic pigment alterations are partly triggered by exposure to elevated ozone but also to reduction of sulphur dioxide emission (Alonso et al. 2001). Beckerson and Hofstra (1979) found that in white bean the chlorophyll loss was greater in the pollutant combination (O_3+SO_2) than from the individual pollutants.

Before 2001 there were not differences in the content of ascorbic acid between NC-S and NC-R clones, but after the desulphurisation device

Table 2: Energy production and annual emission of air pollutants from ŠTPP and average concentration of air pollutants at the rural site Zavodnje before (1996–2000) and after (2001–2003) installation of a desulphurization device at unit 5 (Rotnik and Ribarič Lasnik 2004).

Tabela 2: Proizvodnja energije in letne emisije iz TEŠ ter povprečne koncentracije zračnih onesnažil v ruralnem območju Zavodnje pred (1996–2000) in po (2001–2003) namestitvi odžveplevalne naprave na blok 5 (Rotnik in Ribarič Lasnik 2004).

Annual average values		BEFORE (ā ± std)	AFTER (ā ± std)	[1]p
Energy produced in ŠTTP (MWh)		3884836 ± 191219	4359106 ± 159231	*
Emission of air pollutants from ŠTTP per energy produced (tons/MWh)	SO_2	0.01297 ± 0.00095	0.00414 ± 0.00101	***
	NO_x	0.00273 ± 0.00019	0.00268 ± 0.00016	NS
	CO_2	0.90981 ± 0.01581	0.93878 ± 0.05818	NS
	Dust particulate	0.00041 ± 0.00020	0.00012 ± 0.00002	NS
Average annual values of air pollutants in Zavodnje per energy produced (µg/m^3/GWh)	SO_2	0.00982 ± 0.00128	0.00414 ± 0.00066	***
	NO_2	0.00164 ± 0.00018	0.00122 ± 0.00023	NS
	NO_x	0.00200 ± 0.00029	0.00152 ± 0.00030	NS
	O_3	0.01709 ± 0.00117	0.01679 ± 0.00189	NS
	Dust particulate (mg/m^{2}day/MWh)	0.01462 ± 0.00917	0.01477 ± 0.00943	NS
Average annual values of air pollutants in Zavodnje (µg/m^3)	SO_2	38.2 ± 5.72	18.0 ± 2.65	**
	NO_2	6.40 ± 0.89	5.33 ± 1.15	NS
	NO_x	7.80 ± 1.30	6.67 ± 1.53	NS
	O_3	66.4 ± 5.90	73.0 ± 6.24	NS
	Dust particulate (mg/m^2 day)	56.1 ± 33.5	65.3 ± 43.0	NS

[1] NS – not significant, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

Table 3: Average imission concentrations of air pollutants, air temperature and relative humidity during experimental season before (1996–2000) and after (2001–2003) installation of the desulphurization device at unit 5 (no data in 1998).

Tabela 3: Povprečne imisijske koncentracije zračnih onesnažil, temperature zraka in relativne vlage med trajanjem poskusa pred (1996–2000) in po (2001–2003) namestitvi odžveplevalne naprave na blok 5 (manjkajo podatki za leto 1998).

	BEFORE (ā ± std)	AFTER (ā ± std)	t^{df}, p(1)
Air temperature (°C)	16.5 ± 3.34	17.6 ± 4.44	t^{820}=-3.92 ***
Relative humidity (%)	71.3 ± 10.42	67.8 ± 13.3	t^{820}=4.14 ***
SO_2 (µg/m^3)(2)	23.2 ± 24.4	12.31 ± 13.0	t^{806}=7.36 ***
NO_2 (µg/m^3)(2)	4.46 ± 3.34	4.89 ± 6.33	t^{807}=-1.25
NO_x (µg/m^3)(2)	5.01 ± 3.95	5.77 ± 7.40	t^{807}=-1.89
O_3 (µg/m^3)(2)	72.9 ± 26.9	91.7 ± 19.8	t^{815}=-10.7***
AOT40 (ppb) (May-July)	16,355 ± 6,288	22,565 ± 5,803	t^{6} =-1.39

Legend: (1) df = degree of freedom; t = Student's t-test; * = p<0.05, ** = p<0.01, *** = p<0.001
(2)Source of half an hour's data: Ecological informative system of ŠTPP

Table 4: Ozone injuries on leaves of ozone-sensitive (NC-S) and ozone-resistant (NC-R) clones of the ´Regal´ white clover (*Trifolium repens* ´Regal´), biomass and economic losses before (1996–2000) and after (2001–2003) installation of the desulphurization device at unit 5 (no data in 1998).

Tabela 4: Ozonske poškodbe na listih na ozon občutljivem (NC-S) in na ozon rezistentnem (NC-R) klonu plazeče detelje ´Regal´ (*Trifolium repens* ´Regal´), biomasa in ekonomska ocena škode pred (1996–2000) in po (2001–2003) namestitvi odžveplevalne naprave na blok 5 (manjkajo podatki za leto 1998).

	Clone(5)	BEFORE (ā ± std)	AFTER (ā ± std)	t^{df}, F^{df}, p(4)
Injury class (0-6)	NC-S	3.00 ± 0.56	2.39 ± 1.65	$F^{1,19}$=0.65
	NC-R	0.58 ± 1.38	0.19 ± 0.56	$F^{1,19}$=1.47
Frequency of ozone injuries in experimental period (%)(6)	NC-S	90.0 ± 11.5	83.3 ± 28.8	$F^{1,5}$=0.18
	NC-R	16.6 ± 19.2	11.1 ± 19.2	$F^{1,5}$=0.14
Biomass ratio	NC-S/NC-R	0.87 ± 0.13	0.90 ± 0.09	$F^{9,21}$=0.52
Estimation of economic losses	(%)	13.5 ± 13.5	10.1 ± 8.7	t^{5}=0.38
	(ton/ha) (2,3)	0.33 ± 0.33	0.13 ± 0.11	t^{5}=1.02
	(EUR/ha)(1)	21.4±20.9	8.35±7.24	t^{5}=1.02

Legend: (1) Based on the price for 1 kg hay, which was 0.064 EUR (Statistical information, 281/04 (http://www.stat.si)
(2) Surface of pots were 0.091 m^2
(3) Based on the average harvest for all experiment years – 2 t/ha
(4) df = degree of freedom; t = Student's t-test; F = one-way ANOVA; * = p<0.05, ** = p<0.01, *** = p<0.001
(5) NC-S clone – clone sensitive to ozone; NC-R clone resistant to ozone
(6) Percentage of observations, when ozone injures on leaves were recorded through whole experimental period

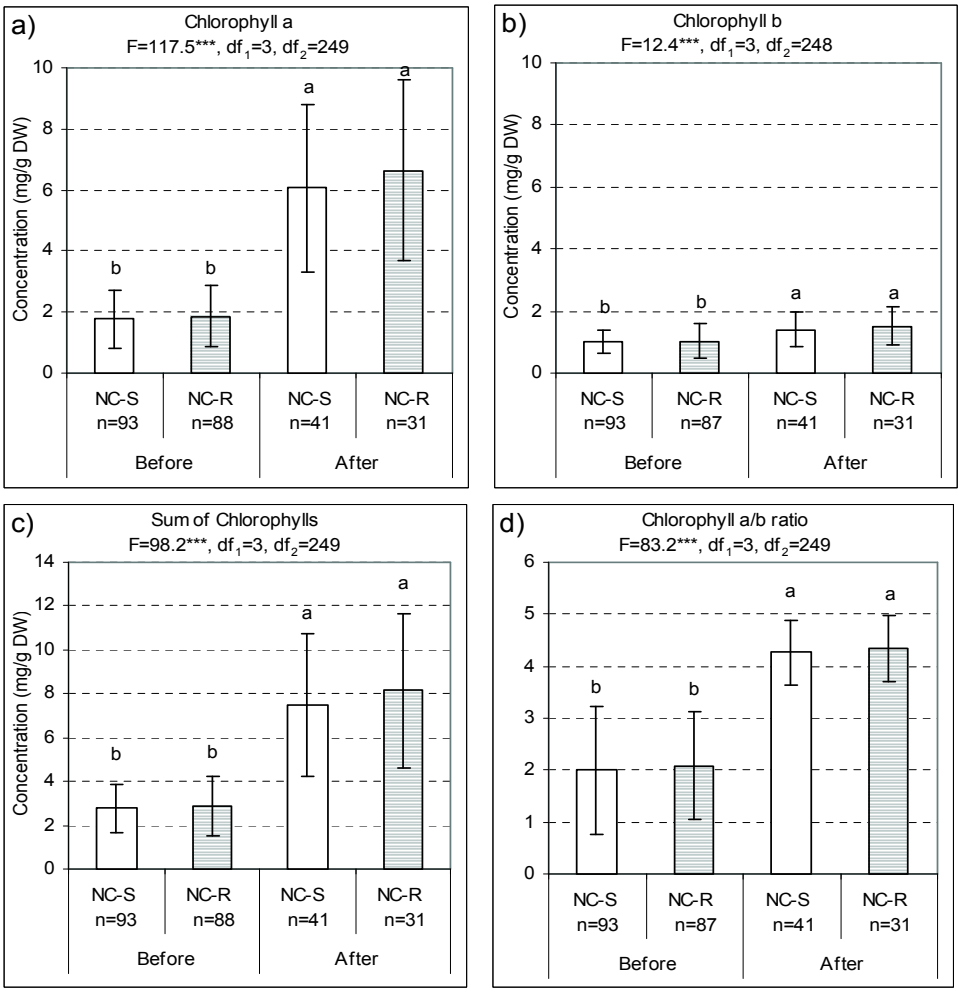

Figure 1a-d: Chlorophyll a (a), chlorophyll b (b), sum of chlorophylls (c) concentration (mg/g DW) and chlorophyll a and b ratio (d) in the leaves of (NC-S) and (NC-R) clones of the ´Regal´ white clover (*Trifolium repens* ´Regal´), exposed in Zavodnje before and after installation of the desulphorization device at unit 5 at ŠTPP.
(Mean ± SD, F=One-way ANOVA, n= number of samples, df=degree of freedom, df_1=df Effect, df_2=df Error, ***, P<0.001, **, P<0.01, *, P<0.05) Different letters indicate significant differences (LSD) among clones.

Slika 1a-d: Koncentracije klorofila a (a), klorofila b (b), vsote klorofilov a in b (c) (mg/g ST) in razmerje klorofil a/klorofil b (d) v listih klonov NC-S in NC-R plazeče detelje ´Regal´ (*Trifolium repens* ´Regal´) v Zavodnjah, pred in po namestitvi odžveplevalne naprave na blok 5.
(Povp. ± SD, F=Enosmerna ANOVA, n= število vzorcev, df=stopnja prostosti, df_1=df učinka, df_2=df napake, ***, P<0.001, **, P<0.01, *, P<0.05). Različne črke označujejo značilne razlike (LSD) med klonoma.

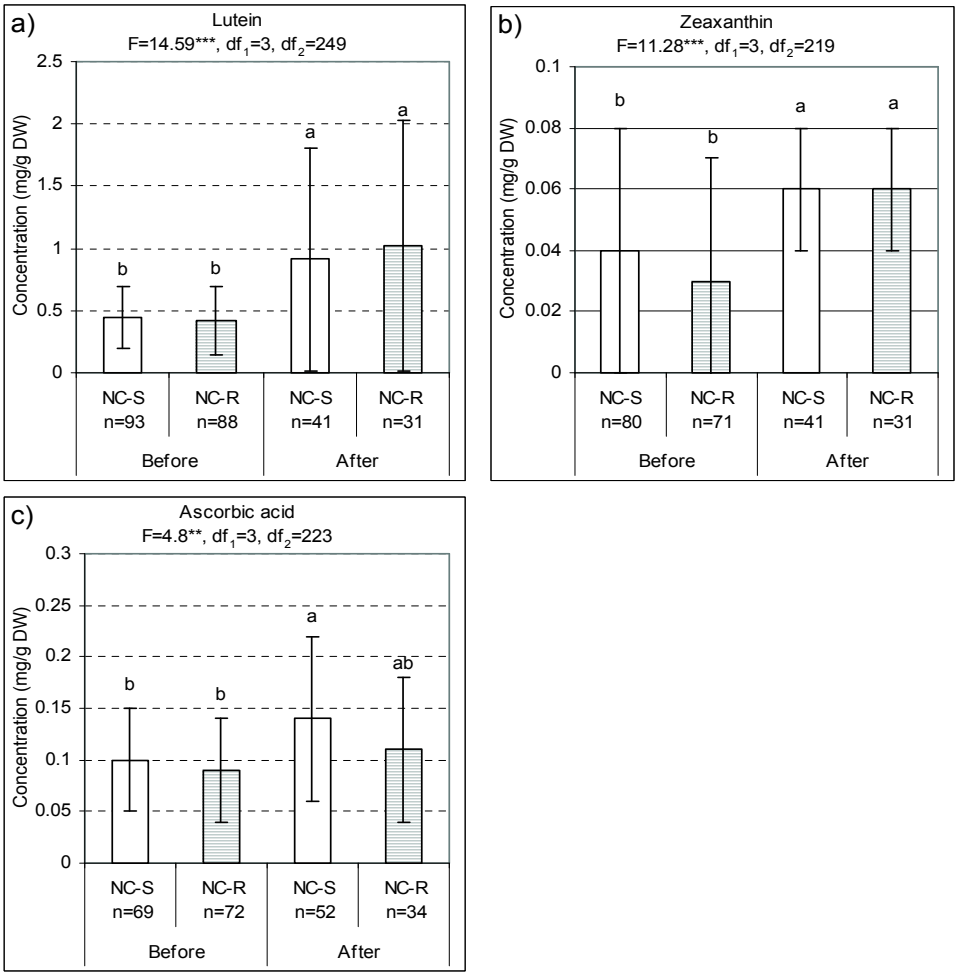

Figure 2a-c: Lutein (a), zeaxanthin (b) and ascorbic acid (c) concentration (mg/g DW) in the leaves of ozone-sensitive (NC-S) and ozone-resistant (NC-R) clones of the ´Regal´ white clover (*Trifolium repens* ´Regal´), exposed in Zavodnje before and after installation of the desulphorization device at unit 5 at ŠTPP (see key for details).
(Mean ± SD, F=One-way ANOVA, n= number of samples, df=degree of freedom, df_1=df Effect, df_2=df Error, ***, P<0.001, **, P<0.01, *, P<0.05) Different letters indicate significant differences (LSD) among clones.

Slika 2a-c: Koncentracije luteina (a), zeaksantina (b) in askorbinske kisline (c) (mg/g DW) v listih klonov NC-S in NC-R plazeče detelje ´Regal´ (*Trifolium repens* ´Regal´) v Zavodnjah, pred in po namestitvi odžveplevalne naprave na blok 5.
(Povp. ± SD, F=Enosmerna ANOVA, n= število vzorcev, df=stopnja prostosti, df_1=df učinka, df_2=df napake, ***, P<0.001, **, P<0.01, *, P<0.05). Različne črke označujejo značilne razlike (LSD) med klonoma.

on ŠTPP started operating, NC-S clone showed higher oxidative stress than NC-R clone (Fig. 2c). The content of ascorbic acid increases with intensity of oxidative stress (Foyer et al. 1997, Šircelj et al. 1997). Also the amount of lutein and zeaxantin (Fig. 2a,b) increased after 2001 at both clones. These results would indicate that the exposure to both O_3 and SO_2 before 2001 might diminish the protection ability of plants to respond to environmental stresses.

Despite of better vitality of plant and higher antioxidant defence ability after 2001, there were no significant differences in the levels of plant injury by ozone between the period 1996-2000 and period 2001-2003, only slight decrease in NC-R clone occurred (Tab. 4). Luwe et al. (1993) ascertained that antioxidants are not always accessible in times of stress. Examples of this are the limited availability of ascorbate in the apoplastic space during attack by the pollutant ozone (the very poor diffusion of ascorbate across the tylakoid membrane that provides ascorbate for the violaxanthin deepoxidase reaction) (Foyer et al. 1990), and the absence of effective antioxidants at the PSII reaction centre to prevent the oxidative damage associated with photo-inhibition (Arora et al. 2002).

The injury class of white clover leaves and frequencies of ozone injuries remained similar after 2001, in the case of NC-R clone a slight improvement can be seen, but not significantly proven (Tab. 4). It could be consequence of smaller synergistic effect of SO_2 and other pollutants (NO_x, NO_2), lower relative humidity and higher temperatures after the year 2000 (Tab. 3). Higher average temperatures and lower relative humidity both induce higher stomatal closure, which makes plants less sensitive to ozone (Benton et al. 2000). The same conclusion can explain why the reduction in SO_2 immission concentration and simultaneous increase in O_3 concentration were reflected in the absence of significant changes in NC-S/NC-R clones biomass ratio (Tab. 4). Moreover, the yield loss of NC-S clone was slightly lower after construction of the desulphurization device, but no statistically significant improvement was observed. The positive effect of lower SO_2 emissions from the ŠTPP on yield losses (in proportion, yield per hectare and costs per hectare) of the white clover clones was also not significant.

Since most of the ozone absorbed through the stomata is decomposed rapidly, chlorosis does not appear to be a primary result of ozone exposure, but rather a secondary effect due to impaired photosynthetic capacity (Heath and Taylor 1997). Actually, there is strong evidence that other factors (phenological stage, nutritional state, co-occurrence with other pollutants and climatic elements) in addition to O_3 pollution influence the biomass ratios (Fuhrer and Achermann 1999, Nali et al. 2009).

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