Gartenbauwissenschaft, 66 (4). S. 164–171, 2001, ISSN 0016–478X. © Verlag Eugen Ulmer GmbH & Co., Stuttgart

Responses of Strawberry to Long-Term Elevated Atmospheric Ozone Concentrations II. Changes of Soluble Phenol Contents in Leaves

Einfluß eines erhöhten Ozongehaltes auf Erdbeere. II. Gehalt löslicher Phenole in Blättern

B. Oertel, N. Keutgen and F. Lenz
(Institut für Obstbau und Gemüsebau der Universität Bonn)

Summary

Strawberry plants of cvs 'Elsanta' and 'Korona' were grown during three months for eight hours five days a week in an atmosphere that contained 65 ppb ozone. Analyses of soluble phenols in leaves indicated that ozone reduced total soluble phenol content, especially contents of agrimoniin, pedunculagin, and GE-56 in both cultivars. The effect of ozone fumigation differed from changes in soluble phenol content related to leaf ageing. The latter process resulted not only in a reduction of almost all soluble phenols but also in an increasing percentage of the flavonoid components. The reduction of gallic acid derivatives and ellagitannins in ozone-treated leaves may be explained by a shortage of their precursor substances phosphoenolpyruvate and erythrose-4-phosphate or by a consumption of soluble phenols in response to ozone stress.

Zusammenfassung

Erdbeerpflanzen der Sorten 'Elsanta' und 'Korona' wurden drei Monate lang fünf Tage pro Woche in einer Atmosphäre von 65 ppb Ozon angezogen. Analysen der löslichen Phenole ergaben, dass Ozon die Konzentration aller löslichen Phenole absenkte, jedoch besonders die von Agrimoniin, Pedunculagin und GE-56 in beiden Sorten. Die Auswirkungen der Ozonbegasung auf die Phenolgehalte unterschieden sich von Blattalterungsprozessen. Letztere bewirkten nicht nur eine Konzentrationsabsenkung sämtlicher löslichen Phenole sondern auch einen steigenden Prozentanteil an Flavonoiden. Die Konzentrationsabnahme der Gallussäurederivate und Ellagitannine in mit Ozon gestressten Blättern kann sowohl auf eine Unterversorgung mit den Vorläufersubstanzen Phosphoenolpyruvat und Erythrose-4-Phosphat zurückgeführt werden als auch mit einem Verbrauch löslicher Phenole in Folge eines Ozonstresses erklärt werden.

Introduction

Ozone is one of the most widespread photochemical air pollutants, which affects plants. It is assumed that once ozone has entered the plant through the stomata, reduced oxygen such as superoxide radicals (O_2^-), hydroxyl radicals ($OH^\cdot$) and hydrogen peroxide (H_2O_2) are formed (LEFOHN 1992, KANGASJÄRVI et al. 1994). Since LAISK et al. (1989) showed that the chloroplast in mesophyll cells is not directly damaged by ozone, it is widely accepted that the first target within the leaf must be the plasma membrane (MORTENSEN and ENGVILD 1995, SANDELIUS et al. 1995). This is strengthened by measurements of antioxidants in the apoplast, which are part of the plant defence system (LUWE and HEBER 1995, POLLE and RENNENBERG 1994), and by investigations, which point to accelerated senescence as a result of ozone exposure (LEHNHERR et al. 1987, NIE et al. 1993, KANGASJÄRVI et al. 1994). Plants generally accomplish the ability to scavenge active oxygen and free radicals generated by ozone through the synthesis of a variety of antioxidant molecules, including phenols such as flavonoids and α-tocopherol, ascorbate, polyamines, glutathione, and carotenoids. Antioxidant enzymes such as superoxide dismutase, catalase, and glutathione S-transferase also play a role in the removal of free radicals or lipid peroxidation products (CONKLIN and LAST 1995). It was the aim of the present study, to focus on the contribution of soluble phenols in strawberry response to ozone stress. In strawberry, such as in other genera of the tribus *Rosoideae* of the *Rosaceae*, ellagitannins represent a major group of phenols. The identification of some of their chemical structures has been reported (OKUDA et al. 1992, OERTEL 1994, MOTOMORI et al. 1995) and a study by HATANO et al. (1989) revealed that ellagitannins are characterised by the ability to scavenge superoxide radicals efficiently. Consequently, soluble ellagitannins in strawberry may be involved in the protection for oxidative stress, especially ozone stress. The present work set out to evaluate the role of gallic acid derivatives and ellagitannins as antioxidants in the defence system against oxidative stress in the strawberry cvs 'Elsanta' and 'Korona'. Analyses were also made to study the effects of a long-term application of 65 ppb ozone on the leaf concentrations on selected phenols at different leaf stages.

Materials and Methods

Plant material

For the ozone applications in 1996 and 1997, 28 strawberry plants (*Fragaria* x *ananassa* Duch.) per year of the cvs 'Elsanta' and 'Korona' were grown as described in a previous paper (KEUTGEN and LENZ 2001). Ozone fu-

migations lasted from May to July 1996 and from April to June 1997, respectively, and one set of plants received an atmosphere that contained 65 ppb ozone during eight hours per day, five days a week in three consecutive months. Leaf samples to determine the soluble phenols were collected in July 1996 and June 1997. Only leaf samples from plants grown in ozone-enriched or charcoal-filtered air were compared, because distinct differences between plants grown in charcoal-filtered and unfiltered air could not be detected (KEUTGEN and LENZ 2001). In July 1996, samples for analyses of leaf carbohydrate contents were taken.

HPLC analysis of soluble phenols

In July 1996, only fully expanded strawberry leaves of the second whorl were analysed, but in June 1997, not completely developed, young leaves of the first whorl, fully expanded leaves of the second whorl and of the fourth whorl were sampled. Leaves of the fourth whorl didn't show clear signs of ageing such as degradation of chlorophyll when harvested. The leaves were sampled at 11.00 a.m. during ozone-fumigation, dried in an oven with circulating air at 40 °C until constant weight, and ground with a Retsch mill type MM 2000 (Retsch, Haan, Germany). The powder was extracted with 50 % (v/v) methanol for 30 min in an ultra-sonic bath at room temperature. After centrifugation the samples were immediately injected onto the analytical HPLC column via a 10 µl loop. The small portion of 50 % methanol and the small injection loop of 10 µl was chosen to avoid methanol dependent peak deformation in the chromatograms.

A high pressure gradient procedure with two HPLC pumps (Waters, Eschborn, Germany) performing a shallow gradient of 0.5 % methanol per minute was chosen for better separation of different tannins. The isocratic dwell time at the beginning of the gradient programme was adapted to the different length of capillaries of different pumps (Waters 501, 515, and 590). By variation of dwell time between 2 and 6 min, the retention time of the main tannin component of strawberry leaves, agrimoniin, was adjusted to 48.9 min, to make chromatograms comparable. The HPLC separation was made using 0.125 % of 85 % o-phosphoric acid in water (solvent A) and methanol (solvent B). The analysis gradient was the following: isocratic with 10 % B for 5 min followed by a linear gradient to 45 % B over 75 min. Methanol was chosen as organic solvent, because the use of acetonitrile led to overlapping of important components. The best adsorbent for strawberry leaf extracts was found to be eurospher 100-C18 5 µm (Knauer, Berlin-Zehlendorf, Germany) formerly called Eurosil Bioselect 100-5 C18 (OERTEL 1994). The column (250 x 4 mm) was maintained at a temperature of 35 °C.

Detection was performed at three wavelengths using the UV detectors Kontron 332 at 280 nm (Kontron, Neufahrn, Germany) and Perkin-Elmer LC-75 at 310 nm (Perkin-Elmer, Überlingen, Germany) as well as a Knauer UV/VIS detector (Knauer, Berlin-Zehlendorf, Germany) with a tungsten lamp at 380 nm. All phenols showed signals at 280 nm. Gallic acid derivatives and ellagitannins showed a very characteristic small absorption at 310 nm, (+)-catechin and proanthocyanidins (flavan-3-ols) could be detected only at 280 nm. Hydroxy cinnamic acids showed intensive signals at 310 nm, kaempferol glycosides showed medium sized and quercetin glycosides signals of normal height at 380 nm.

Agrimoniin, pedunculagin, potentillin, casuarictin, and procyanidin B3 were identified by co-chromatography with authentic samples of these natural products, in accordance to literature data (OERTEL 1994, OKUDA et al. 1992). Calibration of flavan-3-ols was done with (+)-catechin, of gallo- and ellagitannins with gallic acid, of hydroxy cinnamic acids with chlorogenic acid and of kaempferol and quercetin glycosides with rutin (all from Roth, Karlsruhe, Germany). The amounts of these phenols are given in (+)-catechin, gallic acid, chlorogenic acid, and rutin equivalents. The total amount of phenols was defined as sum of all HPLC peaks at 280 nm and calculated approximately in gallic acid equivalents.

Analyses of leaf carbohydrate contents

Leaf samples were collected at the end of July 1996, freeze-dried to constant weight and ground. Starch, glucose, fructose, and sucrose were analysed by sequential enzymatic degradation procedures (CHEN et al. 1997). Total carbohydrate content was regarded as the sum of the measured soluble carbohydrates and starch.

Data analyses

The experimental data were analysed with the SPSS statistical package (SPSS INC., Chicago, USA). For the analyses of leaf phenol contents, a multifactorial ANOVA was performed to evaluate the effects of the ozone fumigation, leaf age and harvest date. A 5 % probability level was accepted to indicate significant differences. For the analyses of leaf carbohydrate contents, the data were tested for normal distribution and variance homogeneity and were compared by Duncan or Wilcoxon tests. For the latter, the probability level was corrected according to Bonferroni.

Abbreviations: GE, gallic acid derivative or ellagitannin; H, hydroxy cinnamic acid derivative; K, kaempferol glycoside; P, phenol; Q, quercetin glycoside.

Results

Soluble phenols

The ellagitannin agrimoniin was the major soluble phenol in strawberry leaves and accounted for 30 to 40 % of total soluble phenols in cvs 'Elsanta' and 'Korona', with the exception of the fourth whorl in cv. 'Korona', where it accounted for about 20 % (Tables 1–4). The two-years study revealed that three months of 65 ppb ozone reduced total phenol concentration, agrimoniin, pedunculagin, GE-54, GE-56, and Q-61 significantly in leaves of the second whorl of cv. 'Elsanta', whereas in cv. 'Korona' only K-69 was lowered (Tables 1–2). The concentrations of most of the soluble phenols differed significantly between 1996 and 1997. In cv. 'Elsanta', only levels of H-7, GE-26, GE-47, GE-50, Q-61 and

K-69 were similar (Table 1). For cv. 'Korona', the concentrations of soluble phenols were slightly more similar between the years. Comparable levels were observed for agrimoniin, potentillin, procyanidin B3, chlorogenic acid, GE-47, GE-54, GE-56, and Q-61 (Table 2).

When responses to ozone fumigation of leaves from the first, second, and fourth whorl in 1997 were evaluated, ozone reduced concentrations of agrimoniin, pedunculagin, GE-54, and GE-56 significantly in cv. 'Elsanta' (Table 3), which was quite similar to the ozone effect on leaves of the second whorl in 1996. In cv. 'Korona', however, a reduction in levels of agrimoniin, pedunculagin, potentillin, chlorogenic acid, GE-50, and GE-56 was observed in addition to the decrease of K-69

Table 1. Combined effects of year of experiment and 65 ppb ozone applied during three months on concentrations of soluble phenols in mg per g dry mass of leaves of the second whorl of the strawberry cv. 'Elsanta' (1. year (1996) control and 1. year (1996) ozone: n = 14; 2. year (1997) control: n = 8; 2. year (1997) ozone: n = 7).
Kombinierte Effekte des Versuchsjahres und 65 ppb Ozon, appliziert für drei Monate, auf die Konzentrationen der löslichen Phenole in mg pro g Trockenmasse von Blättern des zweiten Wirtels der Erdbeersorte 'Elsanta' (1. Jahr (1996) Kontrolle und 1. Jahr (1996) Ozon: n = 14; 2. Jahr (1997) Kontrolle: n = 8; 2. Jahr (1997) Ozon: n = 7).

Phenol	1. year contr.	1. year ozone	2. year contr.	2. year ozone	$P._{main\ eff.}$	$P._{year\ eff.}$	$P._{ozone\ eff.}$	$P._{interactions}$
Total phenols	81.5 ± 26.6	62.3 ± 24.2	111.2 ± 17.8	97.9 ± 18.6	< 0.001	< 0.001	0.035*	0.696
Pedunculagin	3.72 ± 1.12	2.85 ± 0.94	1.34 ± 0.39	0.86 ± 0.17	< 0.001	< 0.001	0.002**	0.535
Procyanidin B3	1.40 ± 0.62	0.93 ± 0.48	2.48 ± 0.55	2.31 ± 0.83	< 0.001	< 0.001	0.114	0.446
Catechin	1.19 ± 0.61	0.89 ± 0.65	4.68 ± 1.62	5.31 ± 0.82	< 0.001	< 0.001	0.883	0.098
Chlorogenic acid	0.28 ± 0.18	0.27 ± 0.18	0.86 ± 0.28	0.86 ± 0.14	< 0.001	< 0.001	0.902	0.938
Potentillin	0.88 ± 0.34	1.05 ± 0.88	1.71 ± 0.28	1.54 ± 0.43	0.006	0.002	0.995	0.375
Agrimoniin	30.9 ± 11.0	23.6 ± 11.3	42.1 ± 5.50	35.9 ± 7.70	< 0.001	0.001	0.039*	0.854
H-7	2.46 ± 0.47	2.19 ± 0.44	2.17 ± 0.25	1.92 ± 0.10	0.047	0.080	0.060	0.960
P-20	1.01 ± 0.61	0.66 ± 0.75	2.94 ± 1.02	3.32 ± 0.60	< 0.001	< 0.001	0.970	0.136
GE-25	0.38 ± 0.28	0.21 ± 0.24	0.85 ± 0.23	0.98 ± 0.12	< 0.001	< 0.001	0.767	0.061
GE-26	1.20 ± 0.58	0.84 ± 0.31	0.87 ± 0.21	0.80 ± 0.25	0.379	–	–	0.662
GE-47	4.41 ± 1.88	3.18 ± 1.50	5.12 ± 0.92	4.29 ± 1.35	0.052	–	–	0.711
GE-50	2.36 ± 1.33	1.68 ± 0.81	2.13 ± 0.70	1.46 ± 0.40	0.073	–	–	0.699
GE-54	4.63 ± 2.02	3.22 ± 1.84	7.68 ± 1.44	6.19 ± 2.83	< 0.001	< 0.001	0.031*	0.951
GE-56	4.06 ± 1.44	2.75 ± 1.54	6.48 ± 1.91	4.55 ± 1.23	< 0.001	< 0.001	0.002**	0.535
Q-61	2.70 ± 0.72	2.05 ± 0.85	2.64 ± 0.53	2.04 ± 0.89	0.049	0.884	0.015*	0.938
K-69	3.16 ± 1.15	2.91 ± 1.23	2.67 ± 1.02	3.00 ± 0.60	0.840	–	–	0.416

The values represent means ± S.D. P-values are the probabilities of the multifactorial ANOVA. Symbols indicate a significant ozone-effect at the 5% (*) or 1% (**) probability level. $P._{year\ eff.}$ and $P._{ozone\ eff.}$ were not calculated, in case $P._{main\ eff.}$ did not indicate a significant main effect.

Table 2. Combined effects of year of experiment and 65 ppb ozone applied during three months on concentrations of soluble phenols in mg per g dry mass of leaves of the second whorl of the strawberry cv. 'Korona' (1. year (1996) control and 1. year (1996) ozone: n = 14; 2. year (1997) ozone: n = 8; 2. year (1997) control: n = 6).
Kombinierte Effekte des Versuchsjahres und 65 ppb Ozon, appliziert für drei Monate, auf die Konzentrationen der löslichen Phenole in mg pro g Trockenmasse von Blättern des zweiten Wirtels der Erdbeersorte 'Korona' (1. Jahr (1996) Kontrolle und 1. Jahr (1996) Ozon: n = 14; 2. Jahr (1997) Ozon: n = 8; 2. Jahr (1997) Kontrolle: n = 6).

Phenol	1. year contr.	1. year ozone	2. year contr.	2. year ozone	$P._{main\ eff.}$	$P._{year\ eff.}$	$P._{ozone\ eff.}$	$P._{interactions}$
Total phenols	77.9 ± 34.0	78.0 ± 8.4	66.3 ± 15.2	66.5 ± 27.7	0.514	–	–	0.998
Pedunculagin	3.43 ± 1.80	3.68 ± 1.43	0.66 ± 0.15	0.62 ± 0.18	< 0.001	< 0.001	0.999	0.527
Procyanidin B3	1.05 ± 0.50	1.00 ± 0.76	0.93 ± 0.31	1.21 ± 0.68	0.834	–	–	0.425
Catechin	1.36 ± 0.72	1.08 ± 0.55	4.99 ± 1.47	4.90 ± 1.62	< 0.001	< 0.001	0.496	0.612
Chlorogenic acid	0.17 ± 0.06	0.27 ± 0.23	0.34 ± 0.12	0.25 ± 0.07	0.056	–	–	0.159
Potentillin	1.38 ± 1.39	0.85 ± 0.38	1.00 ± 0.33	0.92 ± 0.48	0.559	–	–	0.760
Agrimoniin	28.2 ± 15.1	29.4 ± 13.1	20.8 ± 5.6	21.4 ± 10.4	0.195	–	–	0.951
H-7	2.34 ± 1.46	2.59 ± 0.91	1.48 ± 0.12	1.53 ± 0.39	0.025	0.007	0.651	0.758
P-20	0.99 ± 0.77	1.04 ± 0.58	3.51 ± 1.11	3.14 ± 1.13	< 0.001	< 0.001	0.563	0.448
GE-25	0.36 ± 0.34	0.39 ± 0.32	0.90 ± 0.23	0.98 ± 0.37	< 0.001	< 0.001	0.583	0.785
GE-26	1.23 ± 0.56	1.14 ± 0.56	0.64 ± 0.17	0.67 ± 0.30	0.028	0.010	0.599	0.669
GE-47	4.24 ± 2.55	4.86 ± 2.15	2.87 ± 1.00	3.05 ± 1.85	0.087	–	–	0.762
GE-50	2.21 ± 1.19	2.47 ± 1.13	1.21 ± 0.31	1.13 ± 0.60	0.002	< 0.001	0.867	0.401
GE-54	4.23 ± 2.22	4.40 ± 2.81	3.47 ± 1.81	3.37 ± 2.57	0.546	–	–	0.873
GE-56	3.57 ± 1.67	3.74 ± 1.69	3.00 ± 1.13	2.93 ± 1.41	0.420	–	–	0.817
Q-61	3.09 ± 1.36	2.97 ± 1.66	2.00 ± 0.77	1.88 ± 1.10	0.058	–	–	0.998
K-69	3.67 ± 1.17	2.68 ± 1.22	2.74 ± 0.65	1.57 ± 1.11	0.001	0.009	0.006**	0.810

The values represent means ± S.D. P-values are the probabilities of the multifactorial ANOVA. Symbols indicate a significant ozone-effect at the 5% (*) or 1% (**) probability level. $P._{year\ eff.}$ and $P._{ozone\ eff.}$ were not calculated, in case $P._{main\ eff.}$ did not indicate a significant main effect.

seen in the second whorl (Table 4). It can be concluded that agrimoniin, pedunculagin, and GE-56 are reduced in both strawberry cultivars due to a three-months application of 65 ppb ozone. Because agrimoniin is the major soluble phenol in strawberry, total soluble phenol content should be reduced by ozone as well.

The identified phenols were grouped into GEs (gallic acid derivatives and ellagitannins) and flavonoids. Pe-

Table 3. Combined effects of leaf age and 65 ppb ozone applied during three months in 1997 on concentrations of soluble phenols in mg per g dry mass of leaves of the strawberry cv. 'Elsanta' (1. whorl ozone, 2. whorl control, 4. whorl control, and 4. whorl ozone: n = 8; 2. whorl ozone: n = 7; 1. whorl control: n = 3).
Kombinierte Effekte des Blattalters und 65 ppb Ozon, das 1997 für drei Monate appliziert wurde, auf die Konzentrationen der löslichen Phenole in mg pro g Trockenmasse von Blättern der Erdbeersorte 'Elsanta' (1. Wirtel Ozon, 2. Wirtel Kontrolle, 4. Wirtel Kontrolle und 4. Wirtel Ozon: n = 8; 2. Wirtel Ozon: n = 7; 1. Wirtel Kontrolle: n = 3).

Phenol	1. whorl contr.	1. whorl ozone	2. whorl contr.	2. whorl ozone	4. whorl contr.	4. whorl ozone	$P._{main\ eff.}$	$P._{whorl\ eff.}$	$P._{ozone\ eff.}$	$P._{interactions}$
Total phenols	146.3 ± 10.4	144.0 ± 40.3	111.2 ± 17.8	97.9 ± 18.6	48.7 ± 32.9	32.3 ± 10.4	< 0.001	< 0.001	0.218	0.809
Pedunculagin	1.66 ± 0.15	1.39 ± 0.30	1.34 ± 0.39	0.86 ± 0.17	0.56 ± 0.29	0.46 ± 0.16	< 0.001	< 0.001	0.004**	0.179
Procyanidin B3	2.77 ± 0.38	1.98 ± 0.76	2.48 ± 0.55	2.31 ± 0.83	1.03 ± 0.83	0.80 ± 0.18	< 0.001	< 0.001	0.074	0.499
Catechin	4.29 ± 0.93	2.59 ± 0.73	4.68 ± 1.62	5.31 ± 0.82	2.43 ± 1.80	1.74 ± 0.58	< 0.001	< 0.001	0.149	0.080
Chlorogenic acid	0.65 ± 0.10	0.50 ± 0.09	0.86 ± 0.28	0.86 ± 0.14	0.42 ± 0.31	0.27 ± 0.10	< 0.001	< 0.001	0.313	0.656
Casuarictin	4.72 ± 2.73	8.82 ± 9.56	1.44 ± 0.66	1.18 ± 0.51	0.90 ± 0.92	0.21 ± 0.19	< 0.001	< 0.001	0.453	0.222
Potentillin	3.67 ± 0.88	3.84 ± 1.12	1.71 ± 0.28	1.54 ± 0.43	1.06 ± 0.77	0.54 ± 0.28	< 0.001	< 0.001	0.243	0.415
Agrimoniin	52.2 ± 4.9	44.7 ± 9.8	42.1 ± 5.5	35.9 ± 7.7	15.1 ± 12.3	11.2 ± 4.4	< 0.001	< 0.001	0.039*	0.866
H-7	2.00 ± 0.15	1.80 ± 0.44	2.17 ± 0.25	1.92 ± 0.10	1.24 ± 0.65	0.91 ± 0.34	0.994	–	–	1.000
P-20	2.65 ± 0.60	2.19 ± 0.73	2.94 ± 1.02	3.32 ± 0.60	1.87 ± 1.12	1.59 ± 0.35	< 0.001	< 0.001	0.651	0.400
GE-25	1.26 ± 0.43	2.43 ± 2.70	0.85 ± 0.23	0.98 ± 0.12	0.48 ± 0.36	0.33 ± 0.05	< 0.001	< 0.001	0.448	0.357
GE-26	1.30 ± 0.43	1.01 ± 0.26	0.87 ± 0.21	0.80 ± 0.25	0.37 ± 0.28	0.37 ± 0.07	< 0.001	< 0.001	0.135	0.362
GE-47	7.47 ± 1.17	5.83 ± 1.09	5.12 ± 0.92	4.29 ± 1.35	4.43 ± 6.84	1.20 ± 0.41	0.033	0.014	0.923	0.994
GE-50	2.82 ± 0.31	2.56 ± 1.11	2.13 ± 0.70	1.46 ± 0.40	0.84 ± 0.72	0.49 ± 0.22	< 0.001	< 0.001	0.072	0.732
GE-54	9.23 ± 1.33	7.40 ± 0.86	7.68 ± 1.44	6.19 ± 2.83	2.66 ± 2.96	1.16 ± 0.49	< 0.001	< 0.001	0.016*	0.975
GE-56	7.95 ± 0.50	6.17 ± 1.69	6.48 ± 1.91	4.55 ± 1.23	2.14 ± 1.78	1.12 ± 0.48	< 0.001	< 0.001	0.003**	0.671
Q-61	1.16 ± 0.97	2.18 ± 1.07	2.64 ± 0.53	2.04 ± 0.89	1.05 ± 0.94	0.66 ± 0.25	< 0.001	< 0.001	0.982	0.057
K-69	2.34 ± 0.60	2.14 ± 1.11	2.67 ± 1.02	3.00 ± 0.60	2.65 ± 1.22	1.96 ± 1.02	0.319	–	–	0.376

The values represent means ± S.D. P-values are the probabilities of the multifactorial ANOVA. Symbols indicate a significant ozone-effect at the 5% (*) or 1% (**) probability level. $P._{whorl\ eff.}$ and $P._{ozone\ eff.}$ were not calculated, in case $P._{main\ eff.}$ did not indicate a significant main effect.

Table 4. Combined effects of leaf age and 65 ppb ozone applied during three months in 1997 on concentrations of soluble phenols in mg per g dry mass of leaves of the strawberry cv. 'Korona' (1. whorl ozone, 2. whorl ozone, 4. whorl control, and 4. whorl ozone: n = 8; 1. whorl control: n = 7; 2. whorl control: n = 6).
Kombinierte Effekte des Blattalters und 65 ppb Ozon, das 1997 für drei Monate appliziert wurde, auf die Konzentrationen der löslichen Phenole in mg pro g Trockenmasse von Blättern der Erdbeersorte 'Korona' (1. Wirtel Ozon, 2. Wirtel Ozon, 4. Wirtel Kontrolle und 4. Wirtel Ozon: n = 8; 1. Wirtel Kontrolle: n = 7; 2. Wirtel Kontrolle: n = 6).

Phenol	1. whorl contr.	1. whorl ozone	2. whorl contr.	2. whorl ozone	4. whorl contr.	4. whorl ozone	$P._{main\ eff.}$	$P._{whorl\ eff.}$	$P._{ozone\ eff.}$	$P._{interactions}$
Total phenols	111.9 ± 29.6	81.8 ± 26.1	66.3 ± 15.2	66.5 ± 27.7	52.3 ± 30.7	25.9 ± 17.5	< 0.001	< 0.001	0.018*	0.237
Pedunculagin	1.05 ± 0.33	0.78 ± 0.44	0.66 ± 0.15	0.62 ± 0.18	0.68 ± 0.42	0.24 ± 0.14	< 0.001	0.001	0.009**	0.226
Procyanidin B3	1.52 ± 0.64	1.41 ± 0.33	0.93 ± 0.31	1.21 ± 0.68	3.51 ± 2.42	2.20 ± 1.18	0.003	0.002	0.337	0.234
Catechin	6.06 ± 2.78	4.47 ± 1.25	4.99 ± 1.47	4.90 ± 1.62	3.93 ± 2.99	2.00 ± 1.35	0.005	0.006	0.055	0.448
Chlorogenic acid	0.30 ± 0.11	0.23 ± 0.09	0.34 ± 0.12	0.25 ± 0.07	0.25 ± 0.21	0.09 ± 0.09	0.003	0.020	0.006**	0.644
Casuarictin	4.47 ± 3.53	2.55 ± 1.84	1.04 ± 0.74	1.44 ± 0.98	0.38 ± 0.26	0.18 ± 0.35	< 0.001	< 0.001	0.232	0.217
Potentillin	2.11 ± 0.94	1.40 ± 0.56	1.00 ± 0.33	0.92 ± 0.48	0.99 ± 0.73	0.33 ± 0.22	< 0.001	< 0.001	0.010*	0.315
Agrimoniin	35.6 ± 9.9	26.1 ± 7.7	20.8 ± 5.6	21.4 ± 10.4	11.7 ± 7.3	5.4 ± 3.6	< 0.001	< 0.001	0.036*	0.222
H-7	1.67 ± 0.18	1.60 ± 0.21	1.48 ± 0.12	1.53 ± 0.39	1.18 ± 0.50	0.68 ± 0.44	< 0.001	< 0.001	0.096	0.076
P-20	3.98 ± 1.66	3.04 ± 0.97	3.51 ± 1.11	3.14 ± 1.13	1.94 ± 1.42	1.33 ± 0.74	< 0.001	< 0.001	0.084	0.819
GE-25	1.63 ± 0.50	1.30 ± 0.46	0.90 ± 0.23	0.98 ± 0.37	0.80 ± 0.57	0.47 ± 0.35	< 0.001	< 0.001	0.154	0.350
GE-26	1.03 ± 0.42	0.82 ± 0.39	0.64 ± 0.17	0.67 ± 0.30	0.79 ± 0.58	0.55 ± 0.40	0.156	–	–	0.636
GE-47	5.88 ± 2.01	3.93 ± 1.66	2.87 ± 1.00	3.05 ± 1.85	1.46 ± 0.98	0.75 ± 0.66	< 0.001	< 0.001	0.064	0.150
GE-50	2.16 ± 0.77	1.34 ± 0.52	1.21 ± 0.31	1.13 ± 0.60	0.66 ± 0.42	0.22 ± 0.12	< 0.001	< 0.001	0.006**	0.160
GE-54	6.11 ± 2.00	4.05 ± 2.02	3.47 ± 1.81	3.37 ± 2.57	0.94 ± 0.69	0.63 ± 0.72	< 0.001	< 0.001	0.126	0.255
GE-56	5.91 ± 1.80	3.52 ± 1.55	3.00 ± 1.13	2.93 ± 1.41	0.94 ± 0.64	0.54 ± 0.60	< 0.001	< 0.001	0.017*	0.423
Q-61	2.72 ± 1.20	2.38 ± 0.94	2.00 ± 0.77	1.88 ± 1.10	1.08 ± 1.02	0.54 ± 0.61	< 0.001	< 0.001	0.253	0.846
K-69	2.17 ± 0.49	1.83 ± 0.42	2.74 ± 0.65	1.57 ± 1.11	2.49 ± 1.11	1.68 ± 1.17	0.048	0.889	0.006**	0.454

The values represent means ± S.D. P-values are the probabilities of the multifactorial ANOVA. Symbols indicate a significant ozone-effect at the 5% (*) or 1% (**) probability level. $P._{whorl\ eff.}$ and $P._{ozone\ eff.}$ were not calculated, in case $P._{main\ eff.}$ did not indicate a significant main effect.

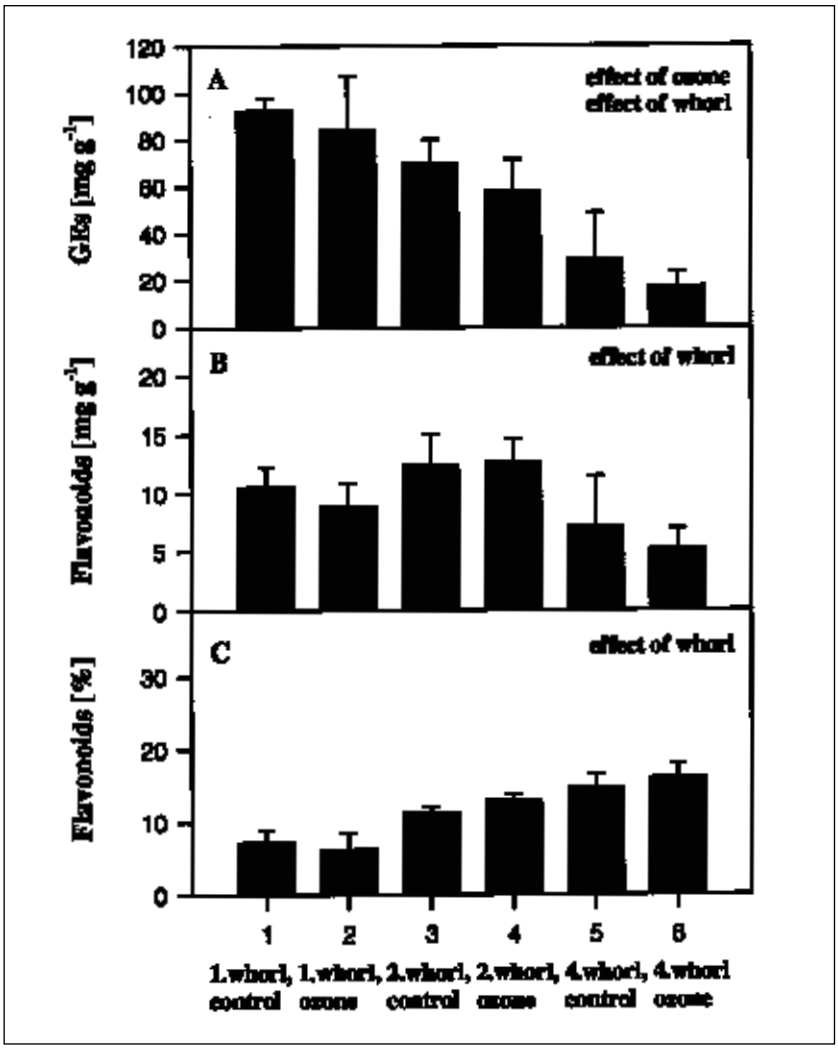


Fig. 1. Effects of 65 ppb ozone and leaf position (whorl) on the concentrations of GEs (ellagitannins and gallic acid derivatives) in mg g^{-1} dry mass (A) as well as flavonoids in mg g^{-1} dry mass (B) and in % of total soluble phenols (C) in leaves of the strawberry cv. 'Elsanta'. The significance of effects was evaluated with a multifactorial ANOVA.
Auswirkungen von 65 ppb Ozon und der Blattposition (Wirtel) auf die Konzentrationen von GEs (Ellagitannine und Gallussäurederivate) in mg g^{-1} Trockenmasse (A) sowie Flavonoide in mg g^{-1} Trockenmasse (B) und in % aller löslichen Phenole (C) von Blättern der Erdbeersorte 'Elsanta'. Die Signifikanz von Effekten wurde mit der multifaktoriellen ANOVA berechnet.

dunculagin, casuarictin, potentillin, agrimoniin, and GE-25 to GE-56 were referred to as GEs, procyanidin B3, catechin, Q-61, and K-69 to as flavonoids. GEs were significantly reduced in cvs 'Elsanta' and 'Korona' by ozone, but flavonoids were lowered only in tendency (Figs 1–2). Nevertheless, when flavonoid contents were expressed in percentage of total soluble phenols, the values of ozone treated plants were in tendency higher (Figs. 1C and 2C).

A comparison of the phenol contents of leaves from the first, second, and fourth whorl showed that the concentrations of most phenols depended on leaf age, except of H-7 and K-69 in cv. 'Elsanta' and GE-26 and K-69 in cv. 'Korona' (Tables 3–4). In addition, the concentrations of casuarictin and possibly potentillin were at least twice as high in young leaves of the first whorl in comparison with leaves from older whorls. These two phenols may represent or may be related to precursor substances for the synthesis of more complex ellagitannins. The dependency of phenol content on leaf age may also explain, why Q-61 was not found to be significantly reduced by ozone in cv. 'Elsanta' when evaluating the 1997 measurements on the different leaf stages. The concentration of Q-61 in the ozone-treated leaves of the first whorl exceeded that of the control by a factor of about 2, but was distinctly lower in ozone-fumigated leaves of the second and fourth whorl (Table 3). When the identified flavonoids were expressed in percentage of total soluble phenols, the values significantly increased in cvs 'Elsanta' and 'Korona' during ageing (Figs 1C and 2C).

Leaf carbohydrate contents

Total carbohydrate contents of leaves from the second whorl were significantly reduced in cv. 'Elsanta' and in tendency reduced in cv. 'Korona' (Table 5). The reduction was due to decreases in starch, glucose, fructose, and sucrose contents, with starch being most, sucrose being least affected.

Discussion

In comparison to control plants of strawberry cvs 'Elsanta' and 'Korona', plant growth of those treated with 65 ppb ozone over 3 months was only slightly affected. 'Korona' didn't show any significant effect, but in cv. 'Elsanta' the ozone treatment tended to induce accelerated leaf senescence (KEUTGEN and LENZ 2001). The larger sensitivity of cv. 'Elsanta' to ozone was also indicated by leaf carbohydrate content, which

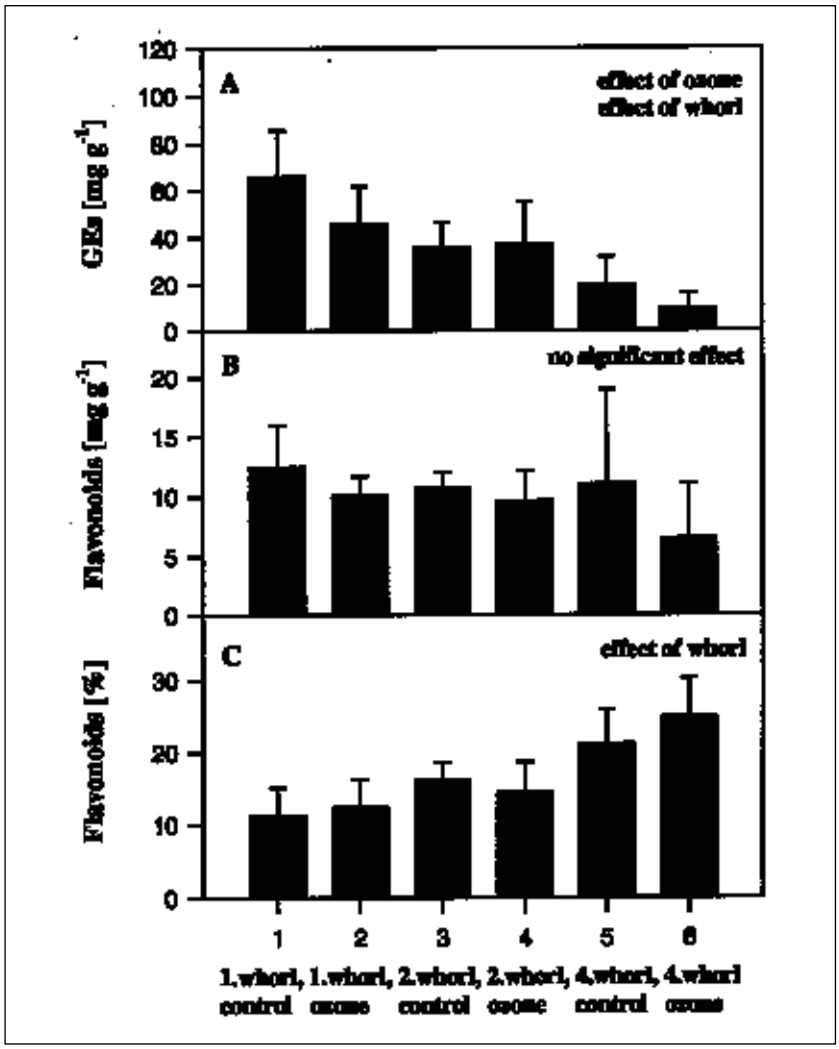


Fig. 2. Effects of 65 ppb ozone and leaf position (whorl) on the concentrations of GEs (ellagitannins and gallic acid derivatives) in mg g^{-1} dry mass (A) as well as flavonoids in mg g^{-1} dry mass (B) and in % of total soluble phenols (C) in leaves of the strawberry cv. 'Korona'. The significance of effects was evaluated with a multifactorial ANOVA.
Auswirkungen von 65 ppb Ozon und der Blattposition (Wirtel) auf die Konzentrationen von GEs (Ellagitannine und Gallussäurederivate) in mg g^{-1} Trockenmasse (A) sowie Flavonoide in mg g^{-1} Trockenmasse (B) und in % aller löslichen Phenole (C) von Blättern der Erdbeersorte 'Korona'. Die Signifikanz von Effekten wurde mit der multifaktoriellen ANOVA berechnet.

Table 5. Responses of carbohydrate contents in % dry mass of leaves from the second whorl of strawberry cvs 'Elsanta' (Els) and 'Korona' (Kor) to a long-term exposure to 65 ppb ozone in 1996.
Reaktionen des Kohlenhydratgehaltes in % der Blatttrockenmasse im zweiten Wirtel der Erdbeersorten 'Elsanta' (Els) und 'Korona' (Kor) auf eine langfristige Begasung mit 65 ppb Ozon in 1996.

	Els, filtered air, no O_3	Els, filtered air, 65 ppb O_3	Kor, filtered air, no O_3	Kor, filtered air, 65 ppb O_3
starch	1.2 ± 0.6 ab	0.6 ± 0.3 b	1.4 ± 0.2 a	0.9 ± 0.2 ab
glucose	1.2 ± 0.4 a	0.8 ± 0.3 a	1.2 ± 0.3 a	0.9 ± 0.2 a
fructose	1.1 ± 0.3 a	0.8 ± 0.3 ab	1.0 ± 0.3 ab	0.7 ± 0.1 b
sucrose	2.4 ± 0.4 a	2.2 ± 1.1 a	2.8 ± 0.7 a	2.8 ± 0.5 a
total	5.9 ± 0.8 a	4.4 ± 1.1 b	6.4 ± 0.9 a	5.4 ± 0.8 ab

The values represent means ± S.D. The number of replicates is 7. In a row, different letters indicate significant differences at the 5% level by Duncan or Wilcoxon tests.

was reduced by 1.5 % in leaves of the second whorl in cv. 'Elsanta', but only 1.0 % in cv. 'Korona' (Table 5). Carbohydrates are the precursor substances of phenols. Without phosphoenolpyruvate and erythrose-4-phosphate, the products of glycolysis and the pentose phosphate pathway, there wouldn't be any phenols (HRAZDINA 1994). This is especially valid for GEs, the dominating group of soluble phenols in strawberry, because gallic acid is an exception that originates from metabolic intermediates of the shikimate rather than the phenylpropanoid pathway (GROSS 1992, 1994, HELLER 1994). Gallic acid is formed by at least two pathways from 3-dehydroshikimic acid with the preferential route depending on the plant species and its developmental stage (GROSS 1994). The reduced soluble phenol contents may be coupled with the reduced carbohydrate content. The reduction of total soluble phenols accounted for about 2 % in leaves of the second whorl of cv. 'Elsanta' in 1996, but there was no reduction in total phenol contents in cv. 'Korona' in

1996. Hence, the reduction of both carbohydrates and soluble phenols was 3.5 % and 1.0 % of dry mass in cvs 'Elsanta' and 'Korona', respectively. This indicates that the contents of further compounds must have been increased to balance for the decreases. Because nitrogen content of leaves in the second whorl increased by about 10.2 % in cv. 'Elsanta' and 8.6 % in cv. 'Korona' (cf. KEUTGEN and LENZ 2001), it might be assumed in accordance with the model of MARGNA that phenol synthesis was reduced in favour of protein synthesis (MARGNA 1977, FEUCHT and TREUTTER 1989).

The larger tolerance of cv. 'Korona' to ozone in comparison to cv. 'Elsanta' is quite obvious when changes in soluble phenols of the second whorl are compared (Tables 1–2). In cv. 'Elsanta' the concentration of total soluble phenols decreased significantly, especially that of agrimoniin, pedunculagin, GE-54, GE-56, and Q-61, whereas in cv. 'Korona' only K-69 was reduced. However, when all measured whorls were taken into consideration, the differences between the cultivars became less distinct (Tables 3–4). Concentrations of agrimoniin, pedunculagin, GE-54, and GE-56 were significantly lowered in cv. 'Elsanta' and of agrimoniin, pedunculagin, potentillin, GE-50, GE-56, K-69, and chlorogenic acid in cv. 'Korona'.

In cv. 'Korona', flavonoid concentrations as a whole (Fig. 2B) were neither affected by ozone nor by leaf age. Nevertheless, in cv. 'Elsanta' an effect of leaf age was detected (Fig 1B), which might be due to the decrease of flavonoid concentrations in the fourth whorl when compared with the second whorl. The relative percentage of flavonoids (Figs 1C and 2C) was not affected by ozone, but ageing resulted in a significant increase in both strawberry cultivars. Flavonoids play an important role in plant defence, e.g. as UV protectans and phytoalexins (FOYER et al. 1994, KANGASJÄRVI et al. 1994). In consequence, the increasing percentage of flavonoids may indicate that carbohydrates were partitioned into the flavonoid synthesis pathway in order to maintain flavonoid concentration in mg g^{-1} leaf dry mass at expense of the gallic acid derivatives and ellagitannins.

HATANO et al. (1989) reported that ellagitannins are able to serve as radical scavengers. We therefore assumed that the concentration of gallic acid derivatives and ellagitannins (GEs) could be related with the ozone tolerance of strawberry cultivars. However, the absolute amount of GEs was always larger in the less ozone tolerant cv. 'Elsanta' than in cv. 'Korona' (Figs 1A and 2A). It is concluded that the concentration of GEs was not related to ozone sensitivity, at least in the studied concentration range. Nevertheless, GEs were significantly reduced by ozone in 'Elsanta' and 'Korona' (Figs 1A and 2A). This may be interpreted as a consumption of GEs in response to ozone stress, but could also be explained by a limitation of carbohydrate supply for the synthesis of GEs.

We acknowledge Prof. T. Okuda, Okayama, for the gift of pedunculagin and potentillin, Prof. T. Yoshida, Okayama, for a sample of agrimoniin, Dr. A. Scalbert, Thiverval-Grignon, for casuarictin, and Dr. L. Kopanski, Bad Honnef, for a sample of procyanidin B3. We are grateful to Mrs. J. Wolter-Sadlers and Mrs. K. Müller for technical assistance, and Deutsche Forschungsgemeinschaft for funding (DFG Le281/26-3 and 39-1).

Literature

CHEN, K., G. HU and F. LENZ 1997: Effects of CO_2 concentration on strawberry. IV. Carbohydrate production and accumulation. Angew. Bot. 71, 183-188.

CONKLIN, P.L. and R.L. LAST 1995: Differential accumulation of antioxidant mRNAs in *Arabidopsis thaliana* exposed to ozone. Plant Physiology 109, 203-212.

FEUCHT, W. and D. TREUTTER 1989: Phenolische Naturstoffe. Ihre Bedeutung für Gartenbau, Land- und Forstwirtschaft. Obst- und Gartenbauverlag des Bayerischen Landesverbandes für Gartenbau und Landespflege e.V., München, Deutschland.

FOYER, C.H., M. LELANDAIS and K.J. KUNERT 1994: Photooxidative stress in plants. Physiol. Plant. 92, 696-717.

GROSS, G.G. 1992: Enzymatic synthesis of gallotannins and related compounds. In: Stafford, H.A. and R.K. Ibrahim (eds), Phenolic Metabolism in plants. Plenum Press, New York, USA, 297-324.

GROSS, G.G. 1994: In vitro studies on the biosynthesis of gallotannins and ellagitannins. Acta Horticulturae 381, 74-80.

HATANO, T., R. EDAMATSU, M. HIRAMATSU, A. MORI, Y. FUJITA, T. YASUHARA, T. YOSHIDA and T. OKUDA 1989: Effects of the interaction of tannins with coexisting substances. VI. Effects of tannins and related polyphenols on superoxide anion radical, and on 1,1-diphenyl-2-picrylhydrazyl radical. Chemical and Pharmaceutical Bulletin 37, 2016-2021.

HELLER, W. 1994: Topics in the biosynthesis of plant phenols. Acta Horticulturae 381, 46-73.

HRAZDINA, G. 1994: Compartmentation in phenolic metabolism. Acta Horticulturae 381, 86-93.

KANGASJÄRVI, J., J. TALVINEN, M. UTRIAINEN and R. KARJALAINEN 1994: Plant defence systems induced by ozone. Plant, Cell and Environment 17, 783-794.

KEUTGEN, N. and F. LENZ 2001: Responses of strawberry to long-term elevated atmospheric ozone concentrations. I. Leaf gas exchange, chlorophyll fluorescence, and macronutrient contents. Gartenbauwissenschaft 66, 27-33.

LAISK A., O. KULL and H. MOLDAU 1989: Ozone concentration in leaf intercellular air spaces is close to zero. Plant Physiology 90, 1163-1167.

LEFOHN, A.S. 1992: Surface Level Ozone Exposures and their Effects on Vegetation. Lewis Publishers, Chelsea, USA.

LEHNHERR, B., A. GRANDJEAN, F. MÄCHLER and J. FUHRER 1987: The effect of ozone in ambient air on RubisCo activity decreases photosynthesis and grain yield in wheat. Journal of Plant Physiology 130, 189-200.

LUWE, M. and U. HEBER 1995: Ozone detoxification in the apoplasm and symplasm of spinach, broad bean and beech leaves at ambient and elevated concentrations of ozone in air. Planta 197, 448-455.

MARGNA, V. 1977: Control at the level of substrate supply - an alternative in the regulation of phenylpropanoid accumulation in plant cells. Phytochemistry 16, 419-426.

MORTENSEN, L. and K.C. ENGVILD 1995: Effects of ozone on ^{14}C translocation velocity and growth of spring wheat (*Triticum aestivum* L.) exposed in open-top chambers. Environmental Pollution 87, 135-140.

MOTOMORI, Y., K. SHIMOMURA, K. MORI, H. KUNITAKE, T. NAKASHIMA, M. TANAKA, S. MIYAZAKI and K. ISHIMARU 1995: Polyphenol production in hairy root cultures of *Fragaria* x *ananassa*. Phytochemistry 40, 1425-1428.

NIE, G.-Y., M. TOMASEVIC and N.R. BAKER 1993: Effects of ozone on the photosynthetic apparatus and leaf proteins during leaf development in wheat. Plant, Cell and Environment 16, 643-651.

OERTEL, B. 1994: HPLC-method to determine complete phenolic patterns in Rosaceous plants containing ellagitannins. Acta Horticulturae 381, 775-781.

OKUDA, T., T. YOSHIDA, T. HATANO, M. IWASAKI, M. KIBO, T. ORIME, M. YOSHIZAKI and N. NARUHASHI 1992: Hydrolysable tannins as chemotaxonomic markers in the Rosaceae. Phytochemistry 31, 3091-3096.

POLLE, A. and H. RENNENBERG 1994: Photooxidative stress in trees. In: Foyer, C.H. and P.M. Mullineaux (eds), Causes of Photooxidative Stress and Amelioration of Defence Systems in Plants. CRC Press, Boca Raton, FL, USA, 199-218.

SANDELIUS, A.S., K. NÄSLUND, A.S. CARLSSON, H. PLEIJEL and G. SELIDEN 1995: Exposure of spring wheat (*Triticum aestivum*) to ozone in open-top chambers. Effects on acyl lipid composition and chlorophyll content of flag leaves. New Phytologist 131, 231-239.

Eingereicht: 20. 1. 2000/9. 5. 2000

Anschrift der Verfasser: Dr. B. Oertel, Dr. N. Keutgen und Prof. em. Dr. F. Lenz Institut für Obstbau und Gemüsebau der Universität Bonn, Auf dem Hügel 6, 53121 Bonn, e-mail: B.oertel@uni-bonn.de; Keutgen_N@yahoo.com