Interaction of Elevated CO_2 and Ozone Concentrations and Irrigation Regimes on Leaf Anatomy and Carbohydrate Status of Young Oak (*Quercus petraea*) Trees

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Young sessile oak (*Quercus petraea*) trees were exposed for one vegetation period in closed environmental chambers in a crossed factorial study on effects to varied CO_2 concentrations, ozone concentrations and irrigation treatments. Elevated CO_2 concentrations (ambient + 350 μmol mol^{-1}) caused a significant increase in biomass production, alterations in leaf anatomy and chloroplast ultrastructure as well as an increase in leaf starch content, as compared to ambient CO_2 concentrations. The effects of elevated O_3 concentrations and drought stress were far less distinct. The leaf starch content was influenced by CO_2 and O_3 in a synergistic manner.

Introduction

In the last few years, two dramatic changes in atmosphere due to human influences are obvious: the decrease of ozone concentration in the stratosphere and the increase of tropospheric concentrations of „greenhouse“ trace gasses, from which CO_2 is accepted as the most important one. The global average CO_2 concentration, currently about 360 μmol mol^{-1}, increases almost steadily by about 0.4% per year, mainly due to combustion of fossil fuels and biomass burning. As a consequence, the CO_2 concentration may nearly double by the end of the 21th century (e.g. Watson *et al.*, 1990; Roeckner, 1992). The anthropogenically enhanced emission of anthropogenic trace gasses is predicted to result in a rise in temperature of the earth's surface. Climate change will manifest itself with an alteration of precipitation patterns or with the frequency of weather extremes. The increasing emission of CO_2 is accompanied by an emission of other trace gasses (Allen, 1990). The large increase in the number of motor vehicles is primarily responsible for the observed increases in annual ozone concentrations.

Despite the great number of investigations dealing with single effects of enhanced CO_2 or ozone concentrations on plants, the joint action of enhanced CO_2 levels and ozone is far less studied. Theoretical considerations suggest that elevated concentrations of carbon dioxide can counteract the noxious effects of ozone and water shortage on plant metabolism, chiefly as a result of decreased stomatal conductance (Allen, 1990; Miller, 1993). In contrast to these considerations, synergistic effects of elevated ozone and CO_2 concentrations have also been reported.

Our experiment was designed as a crossed factorial study on effects of CO_2 concentration, ozone concentration and water supply in a controlled-environment experiment in the climate-controlled chambers of the GSF Forschungszentrum für Umwelt und Gesundheit, GmbH in Neuherberg near Munich. The investigations were carried out on young sessile oak (*Quercus petraea*) trees. In order to reach largely realistic environmental conditions, the climate course of a typical sessile oak stand situated the Palatinate Forest (southwest Germany) was simulated. Forest soil of a typical sessile oak stand was also used as substrate. In terms of its climate preferences, sessile oak covers even shallow soils with unfavourable water supply. In a future, perhaps more warm and dry climate in Central Europe, sessile oak will be of even higher ecological and silvicultural importance than at present (Thomasius, 1991).

In the scope of this multidisciplinary experiment, a number of physiological, biochemical and structural parameters were studied, including photosynthetic gas exchange, chlorophyll fluorescence, photosynthesis pigments, components of the antioxidative system, components of nitrogen

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metabolism, emission of volatile components, cytogenetics, nutrient elements, carbohydrate contents, biometry and leaf anatomy. In this paper, the results of the latter three topics are presented.

Materials and Methods

3-years-old sessile oak (*Quercus petraea* (Matt.) Liebl.) saplings, originating from a natural stand in the Palatinate Forest (forestry office Merzalben), were planted into pots (ca. 14 l) in their natural stand soil a year prior to the experiment. In May 1994 the trees were only moderately fertilized with Hewitt solution and transferred to the environmental chambers of the GSF Forschungszentrum für Umwelt und Gesundheit, GmbH in Neuherberg (four walk-in chambers, each equipped with four identical subchambers) before breaking of the buds. The mild fertilization was repeated several times in the course of the experiment. A detailed survey of the GSF environmental chamber design and the most recent illumination system is given by Payer *et al.* (1986, 1993). Following a randomized selection, 128 trees were assigned to four groups exposed to different fumigation treatments with CO_2 and ozone, as shown in Table I. The low ozone concentration (20 nmol mol^{-1}) was within the range of its natural background concentration. The high ozone concentration of 80 nmol mol^{-1} (at daytime) is frequently reached or even exceeded at the Merzalben stand during summer as half-hour mean value. (Ozone concentrations are monitored by the environmental control station Hortenkopf, located in the immediate vicinity of the Merzalben stand (ZIMEN, 1993)). Additionally, all variants were fumigated continuously with 10 nmol mol^{-1} NO_2.

Furthermore, the irrigation regime was varied. Half of the 128 trees were irrigated continuously (≈ 300 hPa soil water tension; no drought stress), half of the trees were subjected to a four-week drought stress period in July by withholding water supply. The climatic protocol (air temperature and humidity, illumination regime) followed the seasonal patterns of the natural stand, as monitored by the environmental control station Hortenkopf (ZIMEN, 1993).

In August 17th 1994, half of the trees (i.e. 64 trees) were sampled for determination of biometrical and biochemical parameters, as well as leaf anatomy and ultrastructure. The other half of the trees remained in the environmental chambers until November 1994. In this paper, some results of the August sampling are presented.

The following biometrical parameters were measured: (1) Fresh weight (FW) of spring leaves (leaves produced in May) and lammas leaves (leaves produced in early July) per tree; (2) fresh weight of stem and twigs; (3) fresh weight of the roots (not for all 64 trees); (4) mean leaf area of 20 randomly selected spring and lammas leaves; (5) mean leaf thickness of 4–5 randomly selected spring and lammas leaves and (6) specific leaf area (SLA; leaf area per unit fresh weight).

Leaf anatomy and ultrastructure was studied on spring and lammas leaves of six trees per fumigation treatment, respectively. For each tree and leaf generation, four pieces of different leaves were fixed in 2% formaldehyde and 2% glutaraldehyde in a 0.025 M phosphate buffer (pH 7.2), postfixed in 2% OsO_4 in a 0.05 phosphate buffer, dehydrated through an acetone series and finally embedded in Spurr's medium (Spurr, 1969). Semithick leaf cross sections were cut on an

Table I. Fumigation treatments.

Fumigation treatment	CO_2 concentration	O_3 concentration
"Control"	ambient (≈ 370 μmol mol^{-1})	low (20 nmol mol^{-1})
"O_3"	ambient (≈ 370 μmol mol^{-1})	high (80 nmol mol^{-1} at daytime, 40 nmol mol^{-1} at nighttime)
"CO_2"	elevated (ambient + 350 μmol mol^{-1})	low (20 nmol mol^{-1})
"$O_3 + CO_2$"	elevated (ambient + 350 μmol mol^{-1})	high (80 nmol mol^{-1} at daytime, 40 nmol mol^{-1} at nighttime)

ultramicrotome at 1 μm and subsequently stained with a 0.1% toluidine blue solution. Starch was detected by staining the sections with periodic acid-Schiff's reagent (PAS). Leaf morphometry was carried out on several cross sections per each independent leaf by computer-aided image analysis. The percentage of the following tissues was calculated: upper epidermis, palisade parenchyma, spongy parenchyma, vascular tissue, intercellular space, and lower epidermis. For TEM observations, ultrathin slices were cut on an ultramicrotome at 80 nm and subsequently stained in 0.5% uranyl acetate, followed by 2% lead citrate. The ultrastructure of mesophyll chloroplasts was examined with an Zeiss EM 900.

The contents of the carbohydrates starch, sucrose, glucose, and fructose were quantified in spring leaves of almost all 64 sampled trees. The collected oak leaves were immediately frozen in liquid N_2 and stored at −70 °C until they were analysed. The frozen leaves were homogenized, incubated in 80% ethanol at 80 °C for 30 min and centrifuged. The pellet was re-suspended and extracted in 80% ethanol two more times. The three resulting supernatants were combined and concentrated for the determination of the soluble carbohydrates sucrose, glucose, and fructose. Sucrose was hydrolysed to fructose and glucose by an invertase. Glucose was phosphorylated to glucose-6-P using a hexokinase. Fructose was phosphorylated to fructose-6-P using a hexokinase and transformed to glucose-6-P using phosphoglucoisomerase. The resulting glucose-6-P was transformed into 6-phosphogluconate by glucose-6-P dehydrogenase. The simultaneous reduction of NADP was followed spectrophotometrically at 340 nm. The combined pellets were retained for starch analysis. Starch concentration was determined enzymatically by measuring the glucose concentration after amyloglucosidase digest at 60 °C according to Bergmeyer (1974). Additionally, the percentage of starch grains was calculated microscopically on cross sections from both, spring and lammas leaves. Leaf samples were processed as mentioned above, stained with periodic acid-Schiff's reagent (PAS) and the percentage of starch grains was calculated by computer-aided image analysis.

Biometrical, anatomical and carbohydrate data were analysed by simple factorial analysis of variance (ANOVA; SPSS 7.5 for Windows).

Table II. Statistical significance of the biometric data by analysis of variance (ANOVA).

Biometric parameter	Single effects of			First-order interactions of		
	CO_2	Ozone	Irrigation [I]	CO_2 × Ozone	CO_2 × I	Ozone × I
FW spring leaves	** (high > low)	n.s.	n.s.	n.s.	n.s.	n.s.
FW lammas leaves	** (high > low)	n.s.	n.s.	n.s.	n.s.	n.s.
FW total above-ground biomass	*** (high > low)	n.s.	n.s.	n.s.	n.s.	n.s.
FW roots	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
SLA spring leaves	** (low > high)	n.s.	n.s.	n.s.	n.s.	n.s.
SLA lammas leaves	* (low > high)	n.s.	* (wet > dry)	n.s.	n.s.	*
Leaf area, spring leaves	* (high > low)	n.s.	n.s.	n.s.	n.s.	n.s.
Leaf area, lammas leaves	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Leaf thickness, spring leaves	*** (high > low)	n.s.	n.s.	n.s.	n.s.	n.s.
Leaf thickness, lammas leaves	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

* = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; n.s. = not significant.

Results and Discussion

The influence of the factor CO_2 on the biometrical parameters was clearly more pronounced than the two other factors (cf. Table II). The approximate doubling of the CO_2 concentration led to a significant increase in the biomass of spring and lammas leaves (Figs. 1, 2), stems, twigs and the total above ground biomass. The increase in biomass production due to elevated CO_2 concentrations, even under low soil nutrient conditions, is consistent with the findings for woody plants in general (Ceulemans and Mousseau, 1994; Curtis and Wang, 1998) and for oak in particular. Among all biometric effects, the increased formation of lammas shoots and leaves at the elevated CO_2 concentration was the most striking one. Whereas only 50% of the control trees formed a lammas flush, the percentage of trees which developed a lammas flush was 69% in the O_3 treatment, 75% in the CO_2 treatment and 88% in the O_3 + CO_2 treatment. In young sessile oak trees, the formation of lammas shoots occurs more or less regular, although it is clearly stimulated by damage to spring leaves (e.g. as a result of damage due to insect herbivory). Under elevated CO_2 concentrations, the stimulation of lammas shoot formation seems to serve as building of new storage organs for extra carbon. These results are similar to those of Picon and Guehl (cited in Ceulemans and Mousseau, 1994), who also found a stimulation of different flushes in sessile oak, due to elevated CO_2 concentrations.

In contrast to the increase in above ground biomass, root biomass production remained unaffected by doubling of the CO_2 concentration. Therefore, a decrease in the root/shoot ratio was found at elevated CO_2 levels. This result was unexpected, since in the majority of experiments with elevated CO_2 on woody plants reviewed by Ceulemans and Mousseau (1994) an increase of the root/shoot (R/S) ratio was noticed. The increase in R/S ratio is generally more pronounced in low nutrient supply experiments than in high fertilization experiments, and is proposed to form part of an mineral nutrient acquisition strategy on poor forest soils (Ceulemans and Mousseau, 1994). Fertilization effects are unlikely to be the reason for the lack of response of the root biomass production in the present study. However, a decrease in R/S ratio after exposure to elevated CO_2 concentrations was also noticed in young pedunculate oak (*Quercus robur*) by Picon *et al.* (1996) and Vivin *et al.* (1996). Whether the decrease of the R/S ratio in oak trees is a genus-specific phenomenon or related to growth conditions (e.g. pot size; (Arp, 1991; Thomas and Strain, 1991)) remains unclear.

The mean area and thickness (Figs. 1, 2, 7) of the individual spring leaves were also significantly increased by doubling of the CO_2 concentration.

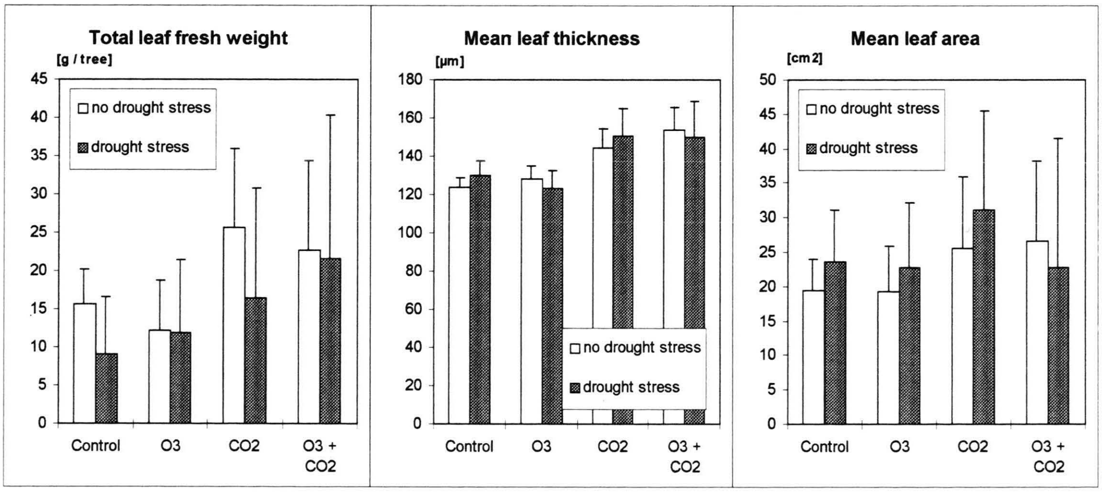

Fig. 1. Arithmetic mean and standard deviation of selected biometrical parameters (total leaf fresh weight per tree, mean leaf thickness and area of individual leaves) of the spring leaves, subjected to different fumigation and irrigation regimes; *n* = 8 trees per treatment.

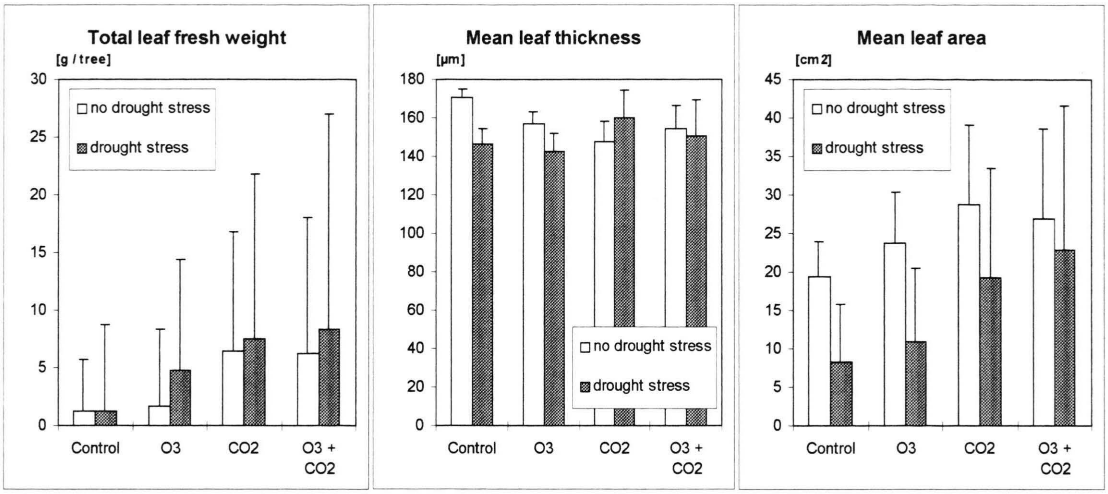

Fig. 2. Arithmetic mean and standard deviation of selected biometrical parameters (total leaf fresh weight per tree, mean leaf thickness and area of individual leaves) of the lammas leaves, subjected to different fumigation and irrigation regimes; $n = 3-8$ trees per treatment.

Due to the increase, both leaf thickness and, as discussed below, an increased starch content per unit leaf area, the specific leaf area (SLA) of spring leaves fumigated with elevated CO_2 concentrations decreased. Similar findings are reported for the majority of woody plants (Ceulemans and Mousseau, 1994). Although in the present study the number of lammas leaves was considerably increased, elevated CO_2 concentrations had no effects on lammas leaf area nor thickness. Probably the latter results were influenced by a chamber effect, because the higher light intensities at the top of chamber resulted in the development of thick „sun“ lammas leaves in all variants.

In contrast to the distinct effects of elevated CO_2 concentrations, the varied ozone fumigation as well as the different irrigation regime had only small effects on the biometrical parameters under investigation. Although the mean lammas leaf area of the drought-stressed trees was lower in all fumigation treatments, the differences were not significant. Only the specific leaf area of lammas leaves of the trees subjected to drought stress was significantly lower as compared to the continuously irrigated trees.

Similar to the results of the biometrical analysis, elevated CO_2 concentrations exerted a significant influence on the percentage of different tissues (Table III). The most pronounced effect was the increase in the percentage of both, palisade and spongy parenchyma in spring and partly in lammas leaves (cf. Figs. 3, 4). This was obviously due to cell enlargement, not to cell proliferation or formation of an additional palisade cell layer (cf. Fig. 7). The absolute volume of individual palisade cells was nearly doubled under elevated CO_2 concentrations (data not shown). The increase in the percentage of the assimilation parenchyma was mainly at the cost of both, the epidermis and the intercellular space. Therefore, the elevated CO_2 concentrations caused not only an increase in leaf volume, but also in cellular biomass per unit leaf volume. In contrast to the above mentioned tissues, vascular tissue remained unaffected. However, the latter may be due to the morphometrical methods used. These results match the scanty reports on effects of elevated CO_2 concentrations on leaf anatomy of woody or herbaceous C_3 plants (Madsen, 1973; Thomas and Harvey, 1983; Vu *et al.*, 1989; Radoglou and Jarvis, 1990). In these studies, an increase in mesophyll thickness was also observed. Mesophyll cell size determines the internal leaf surface area available for the absorption of CO_2 per unit leaf area (Nobel, 1977). Thus, the thickness of the mesophyll may have consequences for the photosynthetic potential.

Ozone fumigation also caused similar, but weaker effects on leaf morphology, as compared to fumigation with elevated CO_2. In spring leaves,

Table III. Statistical significance of spring and lammas leaf morphometrical parameters by analysis of variance (ANOVA).

Biometric parameter	Single effects of			First-order interactions of		
	CO_2	Ozone	Irrigation [I]	CO_2 × Ozone	CO_2 × I	Ozone × I
Spring leaves						
Upper epidermis	** (low > high)	n.s.	n.s.	n.s.	n.s.	*
Palisade parenchyma	*** (high > low)	n.s.	n.s.	n.s.	n.s.	*
Spongy parenchyma	*** (high > low)	* (high > low)	n.s.	n.s.	n.s.	n.s.
Vascular system	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Intercellular space	*** (low > high)	* (low > high)	n.s.	n.s.	n.s.	n.s.
Lower epidermis	** (low > high)	n.s.	n.s.	n.s.	n.s.	n.s.
Lammas leaves						
Upper epidermis	* (low > high)	n.s.	** (dry > wet)	n.s.	n.s.	**
Palisade parenchyma	** (high > low)	* (high > low)	n.s.	n.s.	n.s.	n.s.
Spongy parenchyma	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Vascular system	n.s.	n.s.	n.s.	n.s.	n.s.	*
Intercellular space	*** (low > high)	n.s.	* (wet > dry)	n.s.	n.s.	n.s.
Lower epidermis	n.s.	n.s.	n.s.	*	n.s.	n.s.

* = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; n.s. = not significant.

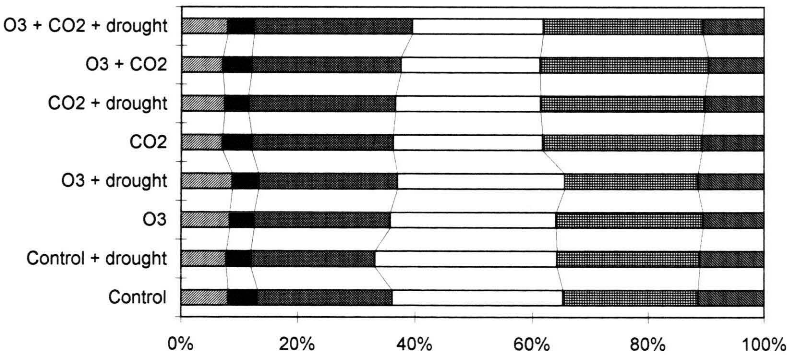

Fig. 3. Percentage of leaf tissues in spring leaves, as subjected to different fumigation and irrigation treatments. Tissues from left to right:

▨ lower epidermis
■ vascular tissue
▩ spongy parenchyma
□ intercellular space
▦ palisade parenchyma
▧ upper epidermis

a significant decrease in the percentage of the intercellular space and an increase of the spongy parenchyma was noticed, while in lammas leaves the percentage of the palisade parenchyma was increased. The varied irrigation regime produced effects only in lammas leaves. Drought stress, ap-

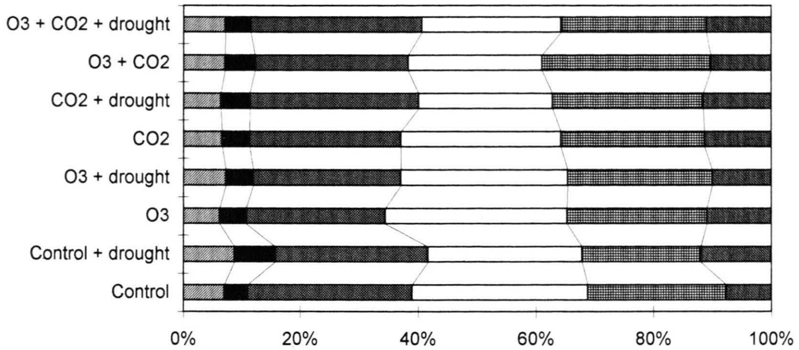

Fig. 4. Percentage of leaf tissues in lammas leaves, as subjected to different fumigation and irrigation treatments. Tissues from left to right:

▨ lower epidermis	■ vascular tissue	▩ spongy parenchyma
□ intercellular space	▦ palisade parenchyma	▧ upper epidermis

plied in the period of lammas leaf expansion, led to an increase in the percentage of the upper epidermis and to a corresponding decrease of the intercellular space. In both cases, the anatomical alterations in oak leaf anatomy may be interpreted as ozone- or drought-induced xeromorphism (Masuch and Kettrup, 1989).

Representative chloroplasts of the oak spring leaves under investigation are depicted in Figure 5. The leaves of the control trees exhibited almost no visual damage symptoms. Chloroplasts of control tree leaves showed a well-developed thylakoid system, some plastoglobules and small starch grains (Fig. 5a). Besides spot and leaf margin necroses, intercostal chlorosis was frequently noticed as visible damage symptom especially on spring leaves of the trees of the O_3 fumigation treatment. Chloroplasts situated in chlorotic leaf sectors exhibited a considerably reduced thylakoid system and huge plastoglobules, while starch grains were mostly small or even absent (Fig. 5b). Similar results have been frequently reported for woody plant leaves, fumigated with ozone (e.g. Sutinen, 1987; Grill *et al.*, 1993; Anttonen *et al.*, 1996). Fumigation with elevated concentrations of CO_2 alone resulted frequently in a slight, diffuse chlorosis of the upper side of the entire leaf. This visual symptom developed in early August, after completion of the lammas shoot formation and was connected with an accumulation of huge starch grains in the chloroplasts (Fig. 5c). A severe disarrangement of the chloroplast endomembrane system due to starch accumulation as described in leaves of several high CO_2 grown herbaceous plants (Cave *et al.*, 1981; Yelle *et al.*, 1989) was, however, never noticed. The trees of the O_3 + CO_2 fumiga-

Table IV. Statistical significance of the carbohydrate amounts (per FW) of spring leaves by analysis of variance (ANOVA).

Carbohydrate	Single effects of			First-order interactions of		
	CO_2	Ozone	Irrigation [I]	$CO_2 \times$ Ozone	$CO_2 \times$ I	Ozone × I
Starch	*** (high > low)	* (high > low)	n.s.	n.s.	n.s.	n.s.
Sucrose	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Glucose	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Fructose	*** (high > low)	n.s.	n.s.	n.s.	n.s.	n.s.

* = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; n.s. = not significant.

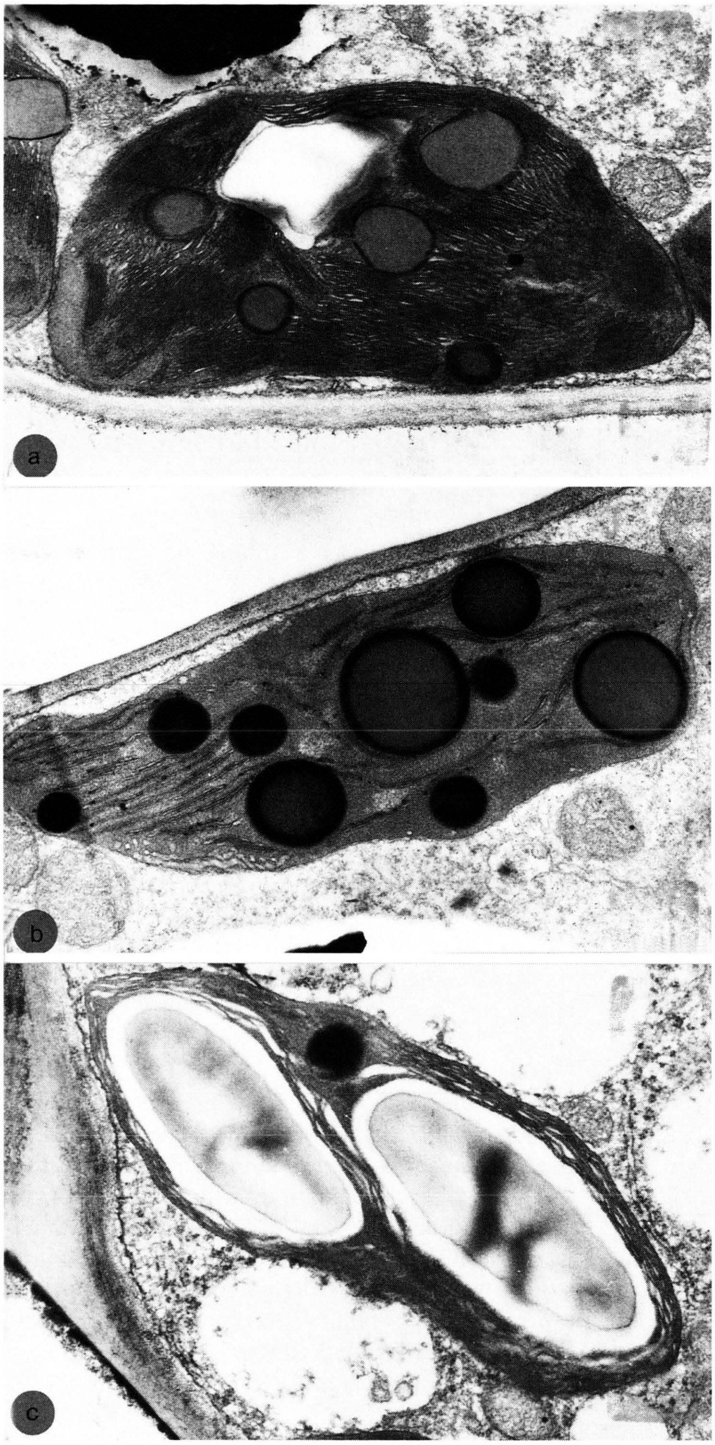

Fig. 5. Ultrastructure of palisade cell chloroplasts in sessile oak spring leaves. a: Chloroplast situated in a green leaf sector of a control tree. Magn.: × 12.000. b: Chloroplast situated in a chlorotic leaf sector of a tree fumigated with elevated O_3 concentrations (O_3 fumigation treatment). Magn.: × 15.000. c: Chloroplast situated of a tree fumigated with elevated CO_2 concentrations (CO_2 fumigation treatment). Magn.: × 9000.

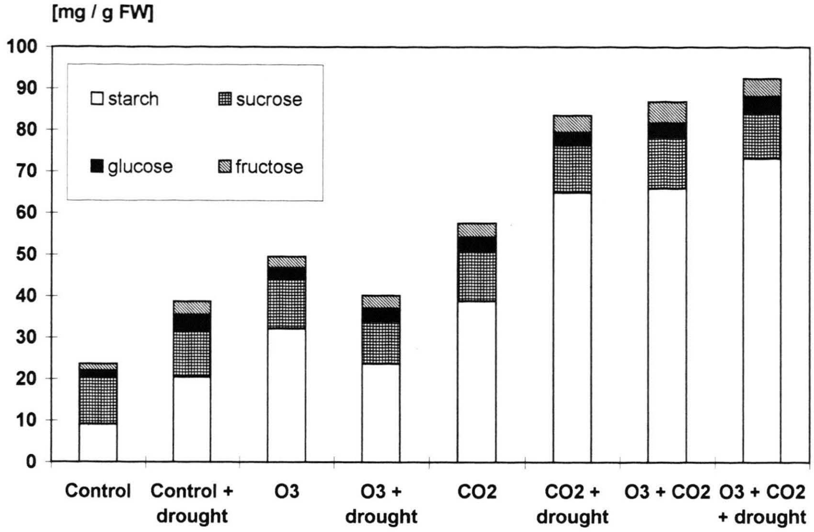

Fig. 6. Arithmetic means of the contents of the individual carbohydrates starch, sucrose, glucose and fructose in sessile oak spring leaves [mg g FW^{-1}]; *n* = 7–8 trees per treatment.

tion treatment developed visual and microscopic damage symptoms similar the symptoms of the elevated CO_2 fumigation treatment. However, additional ozone effects like spot necroses were also clearly visible.

The results of the quantitative carbohydrate analysis in spring leaves are shown in Figure 6. The doubling of the CO_2 concentrations effected a most significant increase of the amount of starch (cf. Table IV). Fumigation with high ozone concentrations also led to a significant increase of the amounts of starch in spring leaves, whereas the varied irrigation regime caused no significant effect. Due to the additive effects of elevated CO_2 and O_3 concentrations, the starch content peaked in the two O_3 + CO_2 fumigation variants. The percentage of starch based on leaf fresh weight was about 5.5% in the mean of the two CO_2 and 7% in the mean of the two O_3 + CO_2 fumigation variants. Starch accumulation was responsible for about 45% and 38% of the respective decrease of specific leaf area in the CO_2 and O_3 + CO_2 fumigation treatments, as compared to the control. Besides starch, the content of fructose was also considerably raised under elevated CO_2, whereas the amounts of sucrose and glucose remained unaffected. In cross sections of leaves fumigated with elevated CO_2 levels, the accumulation of starch was also clearly visible (Fig. 7). The results of the microscopical quantification of starch grains in spring leaf samples were highly correlated with the results of the biochemical starch analysis (data not shown). The microscopical analysis showed that elevated CO_2 concentrations caused starch accumulation in both, spring and lammas leaves (Fig. 8). Similar to the results of the biochemical quantification, the highest percentage of starch grains was noticed in the O_3 + CO_2 treatment.

As a result of long-term elevated CO_2 concentrations, an increase in the starch content of source leaves has frequently been reported in many plant species (e.g. Arp, 1991; Farrar and Williams, 1991). Starch accumulation in source leaves reflects source-sink imbalances with an insufficient demand for carbohydrates in sink organs to balance the enhanced supply in elevated CO_2. Besides CO_2, long term fumigation with elevated ozone concentrations can also lead to starch accumulation in source leaves in many herbaceous and woody plants (e.g. Cooley and Manning, 1987; Gorissen *et al.*, 1993; Willenbrink and Schatten, 1993; Wellburn and Wellburn, 1994). In the case of ozone, starch accumulation may be caused by a disturbed connection between sources and sinks, possibly due to either damage to transport mechanisms across the chloroplast envelope, a reduction in phloem transport capacity or decreased sink-strength.

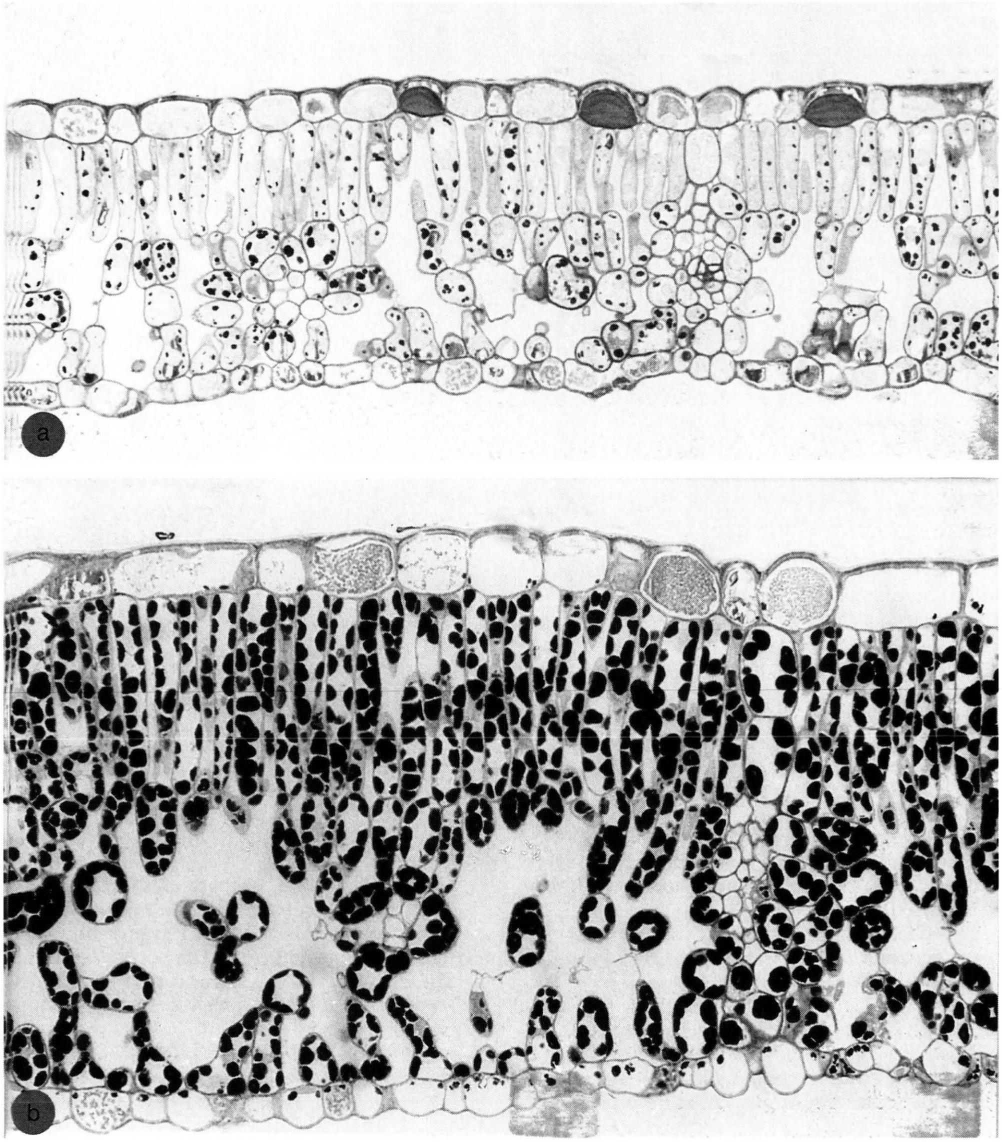

Fig. 7. Cross sections of sessile oak spring leaves. Starch grains stained dark with the PAS procedure. a: Cross section of a control tree leaf. b: Cross section of a leaf of a tree fumigated with elevated CO_2 concentrations (CO_2 fumigation treatment). Note the huge starch grains in the assimilation parenchyma. Both images are magnified in the same scale. Magn.: × 300.

The results of the varied irrigation regime were clearly influenced by the sampling time. During the drought stress phase, a decrease in starch and sucrose contents and a parallel increase in glucose and fructose contents was reported for young oak trees (Epron and Dreyer, 1996). Increases in hexose concentrations are thought to contribute to the osmotic adjustment in drought-stressed plants (Jones *et al.*, 1980). The sampling time in the present study was three weeks after re-watering of the plants. Thus, the lack of differences in the carbohydrate contents between the two irrigation treatments reflects the likely recovery of the plants subjected to drought stress.

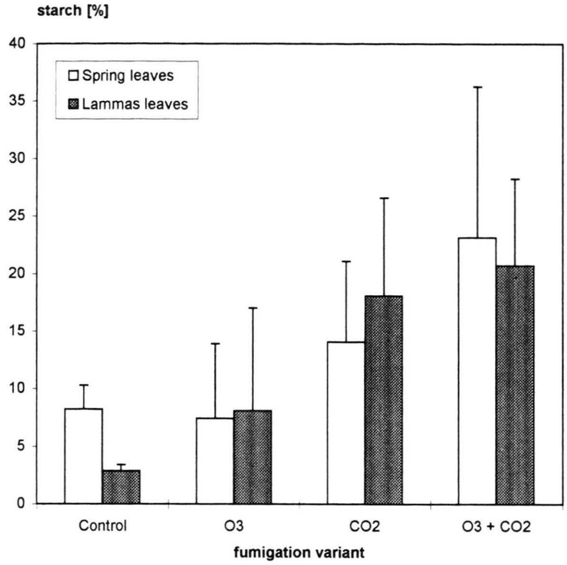

Fig. 8. Arithmetic means and standard deviations of the percentage of starch grains of leaf cross sectional area in spring and lammas leaves. The results of the two different irrigation regimes are combined; n = 4–8 trees per treatment.

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