Role of ethylene in the regulation of cell death and leaf loss in ozone-exposed European beech

ANGELA J. NUNN1, SABINE ANEGG1,*, GUNTER BETZ1, SABINA SIMONS1, GABRIELE KALISCH1, HARALD K. SEIDLITZ2, THORSTEN E. E. GRAMS1, KARL-HEINZ HÄBERLE1, RAINER MATYSSEK1, GÜNTHER BAHNWEG1, HEINRICH SANDERMANN1 & CHRISTIAN LANGEBARTELS1

1Institute of Biochemical Plant Pathology and 2Department of Environmental Engineering, GSF – National Research Center for Environment and Health, D–85764 Oberwiesenthal, Germany and 3Ecophysiology of Plants, TU München, Am Hochanger 13, D-85354 Freising, Germany

ABSTRACT

To test the involvement of ethylene in mediating ozone-induced cell death and leaf loss in European beech (Fagus sylvatica L.), tree seedlings were exposed to proportionally increased or decreased field ozone levels for up to 6 months. Ozone treatment caused cell death and accelerated leaf loss at higher than ambient levels, but had only minor effects at ambient and no effects at subambient ozone levels. The emission of ethylene, the levels of its precursor, 1-aminocyclopropane-1-carboxylate (ACC), and mRNA levels of specific ACC synthase (FS-ACS2) and ACC oxidase (FS-ACO1) isoforms showed a persistent increase and preceded cell death by approximately 2 weeks. Inhibition of ethylene biosynthesis led to reduced lesion formation whereas application of ACC accelerated ozone-induced cell death and leaf loss. Similar results were obtained when adult beech trees were exposed to 2× ozone by a whole tree free-air canopy exposure system. The results suggest a role of ethylene in amplifying ozone effects under field conditions in this major European broad-leaved tree species.

Key-words: Fagus sylvatica L.; abscission; ACC oxidase; ACC synthase; free-air ozone exposure; polyamines; putrescine.

INTRODUCTION

A rapid surge in the foliar production of the gaseous hormone ethylene is a general response in plants confronted with abiotic and biotic stress factors (Abelès, Morgan & Saltveit 1992). The air pollutant ozone is a ubiquitous abiotic stressor that induces ethylene synthesis in plants (Sandermann 1996; Sandermann et al. 1998). The amounts of ethylene and of its direct precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), increase upon ozone exposure of crops and trees as shown in the classical study of Tingey, Standley & Field (1976). In herbaceous plants, a mechanistic connection has been demonstrated between ethylene formation and ozone symptoms, in particular cell death (Mehlhorn & Wellburn 1987; Koch et al. 2000; Overmyer et al. 2000; Moeder et al. 2002). Ethylene is also known to promote senescence-related processes (Kieber 1997; Pell, Schlagnhaufer & Arteca 1997) as well as abscission (Brown 1997), and to mediate alterations in gas exchange which occur under adverse conditions (Taylor, Ross-Todd & Gunderson 1988; Gunderson & Taylor 1991).

It has been shown that ethylene biosynthesis of O3-treated potato and tomato plants proceeds via the normal ACC pathway, namely transcriptional activation of three consecutive enzymes, SAM synthetase, ACC synthase (ACS) and ACC oxidase (ACO; Schlagnhaufer, Arteca & Pell 1997; Tuomainen et al. 1997; Moeder et al. 2002). It is one of the fastest biochemical responses to this air pollutant, and involves the sequential activation of ACS and ACO isoforms at the transcript level (Moeder et al. 2002). Only limited knowledge is available regarding the mechanisms of ozone- and pathogen-induced cell death in trees. Ozone sensitivity of hybrid poplar correlated with defective salicylate and jasmonate signalling (Koch et al. 2000). Short-term induction (24 h) of ethylene levels together with increases in transcript abundance for ACS and ACO isoforms at the transcript level (Moeder et al. 2002). In addition to increases in ethylene emission, levels of free and conjugated ACC were elevated in O3-treated Norway spruce saplings; however, in contrast to the transient nature of ethylene and ACC peaks in herbaceous plants which only last for hours, the responses in coniferous trees were shown to be remarkably persistent (Van den Driessche & Langebartels 1994; Langebartels et al. 1997). In line with these observations, Chen & Wellburn (1989) reported that acid mist treatment resulted in enhanced ethylene emissions as well as in elevated ACC contents for several months in Red spruce and Norway spruce.

Ethylene has been postulated to be involved in regulating the spread of cell death during the hypersensitive response following pathogen attack (Ward et al. 1991; Greenberg 1997) and during ozone-induced cell death in

Correspondence: Dr Christian Langebartels. Fax: +49 89 3187 3383; e-mail: langebartels@gsf.de
* These authors contributed equally to this work.

© 2005 Blackwell Publishing Ltd
herbaceous plants (Langebartels & Kangasjärvi 2004). Inhibition of ethylene biosynthesis or perception has led to marked reductions in ozone-induced cell death (Mehlhorn & Wellburn 1987; Schlagnhauser et al. 1997; Moeder et al. 2002). Activation of ethylene biosynthesis was shown to be one of the fastest and most obvious biochemical responses to ozone, with (transient) responses after 1–5 h and symptom development within hours to days in herbaceous crops. The role of ethylene in cell death and leaf loss in trees in which visible symptoms become obvious after weeks to months (‘memory effect for ozone’; Langebartels et al. 1997) is largely unexplored. The objectives of the present study were therefore: (1) to analyse the persistence of ethylene induction, as well as cell death and leaf loss in dose–response studies with proportional-to-ambient ozone levels; (2) to block or increase ethylene biosynthesis in ozone-exposed beech seedlings and in trees as juvenile trees may distinctly differ in their responses from mature individuals; and (3) to monitor ethylene formation and cell death responses in adult beech trees exposed to ozone under free-air canopy exposure.

MATERIALS AND METHODS

Plant material and conditions of treatment in controlled-environment facilities

Beech seedlings (Fagus sylvatica L., 81017, forest department Siegsdorf, 800 m a.s.l.; or 81024, Kaufbeuren/Krümbach, Southern Bavaria) were obtained from the Bayerische Landesanstalt für forstliche Saat- und Pflanzenzucht, Laufen, and were cultivated in the nursery of the institute before experimentation. Plants were used for exposure studies when they were 3–4 years old (mean height 60–80 cm). To avoid chamber effects, the position of the plants was randomly varied weekly.

Experiment 1 was performed in four GSF controlled-environment growth rooms (EPOKA chambers; 3 m × 3 m each; Thiel et al. 1996) whereas experiments 2 and 3 were conducted in a controlled growth cabinet (2.50 m × 2.50 m) described by Langebartels et al. (1991) and experiment 4 in a glass house. Each growth room contained four individual Plexiglass chambers (1.1 m × 0.9 m × 0.8 m) which were each dedicated to a different treatment. Meteorological data (temperature, relative humidity, irradiance) as well as ozone and NOx concentration data had been collected at an experimental station in Schönenbuch, Switzerland, during the growing season of 1990, and were then used as 2 h mean values in all exposure chamber experiments (Lippert et al. 1996). Plants were continuously treated with proportionally increased or decreased ozone concentrations (24 h d⁻¹, 7 d per week) as described below. Experiments lasted for 7 weeks (experiments 2–4) or the whole growing season from March to September (experiment 1), and the dates of the ‘standard’ year 1990 are given in the legends of Figs 1, 3, 4 and 6. Photoperiods were between 11 and 15 h according to the time of year. Ozone was generated by electrical discharge in dry oxygen and continuously monitored using a computer-controlled system and a UV-type ozone analyser (Payer et al. 1993; Thiel et al. 1996). All air entering the chambers was filtered five-fold through particle, Purafil and activated charcoal filters (Langebartels et al. 1997).

Experiment 1

Approximately 120 2-year-old-beech seedlings were planted in autumn into 5 L pots containing a sandy loam (A-horizon of a Rendzina). The plants were well irrigated and fertilized according to Lippert et al. (1996). They were placed into the GSF controlled-environment chambers in mid-March, before bud break. Following acclimation for 14 d at 7 °C day and 2 °C night temperatures, relative humidity of 70–80% and a day length of 11 h, the climate and ozone values of the field station were simulated from 28 March to 23 September (bud break to leaf loss). Figure 1 shows the applied four ozone levels (2 h means) for three representative days, 10–12 August 1990. In addition to the ambient field O₃ levels (1 × ozone), the latter were proportionally increased by 50% (1.5 × ozone) or 100% (2 × ozone), or were decreased by 85% (0.15 × ozone). 10 plants were randomly allocated to each of the 16 treatment chambers (four replicate treatments). Maximum photosynthetic photon flux density (PPFD) at the top of the canopy averaged 1000 μmol m⁻² s⁻¹ PPFD (Thiel et al. 1996). Irrigation

![Figure 1. Daily time course of ozone levels (experiment 1). Ozone and climate data were recorded at the study site of Schönenbuch, Switzerland in 1990 and were used for all indoor chamber experiments. Three representative days of August 1990 are given as an example. Symbols: (△) 0.15 × ambient ozone; (○) ambient ozone; line: field values; (▲) 1.5 × ambient ozone; (●) 2 × ambient ozone.](image-url)
and fertilization treatments were as described by Lippert et al. (1996).

Experiments 2 and 3
Twenty-five 1-year-old beech seedlings were individually planted into 10 L pots containing a sandy loam and quartz sand (2 : 1, v/v) in May. Plants were cultivated in a greenhouse during two growing seasons, and were kept in the nursery of the institute during the winter period. In June, after the leaves had fully developed, the seedlings were transferred into controlled-environment cabinets. 10 plants were assigned randomly to two ozone treatments (1 × ambient, or 2 × ozone, proportionally added ozone; two replicate treatments) for 44 d from 6 June to 25 July. The plants were well watered and fertilized regularly (Lippert et al. 1996). Maximum PPFD at the top of the canopy averaged 190 μmol m⁻² s⁻¹ PPFD (Langebartels et al. 1991); light was provided by fluorescent tubes. In experiment 3, seedlings were sprayed five times with the ethylene precursor ACC (1 mM; Sigma, Deisenhofen, Germany) or with the biosynthesis inhibitor aminoethoxyvinyl glycine (AVG; 1 mM; Sigma) during a 6-week period from end of May to mid-July. Treatment conditions and design of the experiments were similar to experiment 2.

Experiment 4
Eighteen 4-year-old beech saplings were individually planted in autumn in 10 L containers with standard substrate and sandy loam (1 : 1, v/v). The plants were kept in the nursery of the institute during the winter period, and were transferred to the greenhouse at the beginning of September. Plants were treated with 180–200 nL L⁻¹ ozone (8 h d⁻¹, 0900 to 1700 h) or pollutant-free air for up to 7 weeks. Leaves were collected for molecular analyses at eight time points after the onset of exposure (0 h, 2, 7, 15, 24, 32, 39 and 46 d).

Field experiments
In addition to the natural ambient exposure to ozone adult beech trees were continuously exposed to ambient or 2 × ambient ozone at the Kranzberg free-air exposure site (Nunn et al. 2002; Werner & Fabian 2002). Ozone was generated from oxygen-enriched air and spread through a tubing system across the joint canopy (about 1500 m²) of the adult trees. Five individual trees were selected in 2 × ozone and another five trees in the ambient-ozone canopy. Leaf samples (n = 5) for the determination of biochemical parameters were taken from a shade and a sun branch of each individual tree at 2-week intervals from May 2000 to August 2001. On three separate branches, 1 mM ACC (0.01%, v/v Tween 20) was sprayed until run-off three times every 10–14 d to three twigs with 45–60 leaves each. The twigs were covered with Teflon bags during the 2 h treatment periods. Three independent twigs from the 2 × ozone canopy received control treatments with 0.01% (v/v) Tween 20.

Determination of ethylene emission and ACC contents
Ethylene emission from leaves was measured by placing one or two intact, detached leaves (approximately 0.2 g), wrapped in moist filter paper, in a glass tube which was sealed with a silicon septum (Tuomainen et al. 1997). Following incubation for 1 h in the dark at room temperature, 1 mL of gas was withdrawn with a disposable syringe. Ethylene was analysed using a Perkin Elmer Autosystem XL gas chromatograph (Überlingen, Germany) with a Porapak Q column (80–100 mesh, 0.92 μm × 3 mm) and flame ionization detector (Langebartels et al. 1991). Column, injector and detector temperatures were at 50, 150 and 200 ± 0.5 °C, respectively. The retention times for ethylene and ethane were 0.75 and 1.1 min, respectively.

Determination of polyamine contents
Polyamines were extracted by adding 1.25 mL of 5% (v/v) perchloric acid to 0.1 g of homogenized leaf material (Navakoudis et al. 2003). After incubation for 1 h and centrifugation for 10 min (20 000 g, 4 °C), 15 μL of the supernatant were mixed with 360 μL 0.1 M NaHCO₃, 10 μL acetic acid and 200 μL 0.1 mM 9-fluorenylmethylchloroformate (FMOC) in acetone. The samples were incubated for 5 min at room temperature and subsequently for 10 min at 50 °C. After cooling down on ice, 300 μL of MeOH were added, and 20 μL used for high-performance liquid chromatography (HPLC) analysis. Polyamines were separated on a Spherisorb ODS II column (5 μm, 250 × 4.6 mm, Bischoff, Leonberg, Germany) with a MeOH/water gradient (0–30 min 80–100%; 30–37 min 100%) using a Merck/Hitachi HPLC system (pump 16200, solvent delivery system HS 2000, integrator D 2500; Merck, Darmstadt, Germany) equipped with a spectrofluorimeter (RF 551, Shimadzu, Duisburg, Germany) at excitation and emission wavelengths of 260 and 313 nm, respectively.

Isolation of RNA and quantification of gene expression
The protocol of Kiefer, Heller & Ernst (2000) was applied for total RNA extraction because of the high amounts of phenolic compounds in beech leaves. After DNase treatment (RQ1 Dnase; Promega, Madison, WI, USA) and ethanolic precipitation, total RNA was quantified photo metrically and equal amounts were transcribed to cDNA by means of SuperScript II™ reverse transcriptase and an Oligo(dT)₁₂–₁₈ primer according to the manufacturers protocol (Invitrogen, Carlsbad, CA, USA).

The expression of the analysed genes was assayed by quantitative real-time polymerase chain reaction (PCR) using an AbiPrism 7700 (Applied Biosystems) apparatus. PCR reactions mixtures (25 μL) contained 6 ng cDNA, 5 pmol of each specific primer and 12.5 μL ABsolute™ QPCR SYBR® Green Mix (ABgene, Epsom, Surrey, UK). Specific gene probes for ethylene biosynthetic genes (FS-
ACSI, acs1, AJ420188; FS-ACS2, AY705445; FS-ACOI, aco1, AJ420190), the receptor gene FS-ETRF1 (erf1, AJ606475) and the small subunit of ribulosebisphosphate carboxylase (FS-rbcS, AY705444) were designed for each sequence. Primer pairs were as follows: ACS1: 5'-GGTCTCTCAGTGCTCTG-3' and ACS1R: 5'-CTGCTCTTTGAGTAGGTGAC-3', generating a probe from nucleotides 293–392; ACS2, ACS2F: 5'-TCTAAAC CGCAGATGCTG-3' and ACS2R: 5'-TGGTGAGG GCACAAGAAAG-3', nucleotides 90–189; ACO1, ACO1F: 5'-CTGCTTGGATCTCTTACTC-3' and ACO1R: 5'-AAATGAATGCGCATAGGG-3', nucleotides 539–640; ETR1, ETR1F = 5'-GGTTTTGATTCTC GTGAAAGAGCA-3' and ETR1R: 5'-CTGGTGGGATCATCTTACTC-3', nucleotides 339–436; ETRF1, ETRF1F: 5'-AGCGCTGTAGCGATTGAC-3' and ETRF1R = 5'-TCTTGCACAAGGACCTCTC-3', nucleotides 116–215; rbcS, rbcSF = 5'-ATGATGGACGTTACTGGGTG-3' and rbcSR: 5'-GGATAAGTCTTACTGCGCC-3', nucleotides 363–462. After enzyme activation (95 °C 15 min) 40 PCR cycles (95 °C 15 s, 60 °C 1 min) were performed. The uniform threshold cycles (C\textsubscript{T} values) were converted to relative gene induction values assuming a doubling of PCR template in every cycle during the exponential phase.

Symptom assessment and gas exchange measurements

Seedlings and tree branches were visually assessed every 2–4 d for chlorotic symptoms and cell death (in percentage affected leaf area). Leaf loss was expressed as percentage of leaves remaining on the seedlings or branches. Measurements of net photosynthesis and stomatal conductance were made using portable systems equipped with an infra-red gas analyser (LCA-2, Parkinson leaf cuvette; ADC, Hoddesdon, Herts., UK; COP-130, Walz, Effeltrich, Germany) under ambient light and ozone (1 ×) conditions. Measurements were performed on five to 10 fully expanded leaves per treatment. AOT40 values were calculated according to Fuhrer, Skárby & Ashmore (1997) as the sum of hourly mean ozone concentrations exceeding 40 nL L\textsuperscript{-1} during daylight hours, namely for global radiation above 50 W m\textsuperscript{-2}.

Statistical analysis

Experiments with juvenile trees were performed with four to 10 replicate samples per test condition. When indicated, the Tukey multiple range test was used to test for differences among treatment means (at \( P < 0.05 \); Statgraphics software; STSC, Rockville, MD, USA). Annual courses of ACC and putrescine in leaves of adult trees were tested using the repeated measures module of the general linear model (GLM; SPSS, 12.0; SPSS Inc., Chicago, IL, USA) employing a nested design for crown region and ozone treatment.

RESULTS

Ozone induction of ethylene formation and cell death in dose–response studies with beech seedlings

Treatment of beech seedlings with increased (2 × proportional-to-ambient) ozone levels led to spot-like cell death as illustrated in Fig. 2. Typically, ozone injury occurred in intercostal areas of the upper leaf surface and spread over 70% of the total leaf area during the course of the study (experiment 1). Once initiated, the lesions developed with increasing rates (cf. leaves on day 27 (Fig. 2b) and day 33 (Fig. 2d).

Season-long exposure with 1.5 × or 2 × proportional-to-ambient ozone had significant effects on the development of cell death (Fig. 3a) and leaf loss (Fig. 3b). In early July, the first ozone-induced changes in leaf appearance were observed as slight discoloration and ‘bronzing’ (not shown). Formation of cell death spots started between mid-July and beginning of August, shortly after a period of ozone concentrations above 80 nL L\textsuperscript{-1} in the 1 × ambient-ozone treatment (Fig. 3a). The leaf area covered with cell death increased in parallel in the 1.5 and 2 × treatments, and reached maximum values of approx. 40 and 45%, respectively, by mid-August. Ambient ozone led to delayed and reduced symptom development which reached 30% of the leaf area only after mid-September. Minor symptoms occurred in the treatment with 0.15 × ambient ozone levels (Fig. 3a).

As shown in Fig. 3b, the percentage of leaves remaining on the trees began to decline under the 2 × ozone treatment at the end of August. Leaf loss exhibited a dose–response similar to that in lesion development which was initiated 2–3 weeks earlier in the trees treated with twice-ambient ozone. Seedlings held under the two elevated ozone regimes showed a 50% leaf loss 7–9 d earlier than did seedlings at 1 × and 0.15 × ozone levels (Fig. 3b).

To analyse ozone-induced ethylene emission from beech seedlings, plants were exposed for 44 d to 1 × or 2 × ambient levels (experiment 2). This treatment included a period of ozone levels above 80 nL L\textsuperscript{-1} with maxima at 125 nL L\textsuperscript{-1} in the ambient ozone treatment. Twice-ambient ozone caused a marked stimulation of ethylene emission that started 5 d after the onset of treatment, reaching maximum ethylene levels (60-fold increase) on day 28 of exposure (Fig. 4a). Two exposure periods with high ambient levels above 100 nL L\textsuperscript{-1} resulted in large peaks of ethylene emission in the 2 × ozone treatment, whereas trees exposed to ambient ozone showed very minor increases in ethylene emission. When the kinetics of ethylene induction was followed for 2 d in detail, it became evident that ethylene emission peaked approx. 10 h after the onset of exposure (i.e. between 2000 and 2400 h) and remained elevated until the following day (data not shown). Exposure to 2 × ozone also caused a parallel increase in the levels of ACC, the direct precursor of ethylene, in free and conjugated form, whereas only minor responses were found in the 1 × ozone treatment (data not shown).
Cell death and leaf loss occurred 19 and 23 d, respectively, after the onset of treatment in seedlings exposed to 2 × ozone (Fig. 4b & c). At the end of the experiment, both parameters reached approximately 50%. In contrast, trees at ambient ozone did not develop any symptoms or shedding of leaves. Ethylene emission (Fig. 4a) steeply decreased when the damaged cell death area (Fig. 4b) reached approximately 25%, suggesting that ethylene formation was inhibited at this stage. The levels of free and conjugated ACC, on the other hand, continued to increase until day 44, reaching levels of 100 and 600 nmol g⁻¹ fresh weight, respectively, equivalent to 200- or 60-fold increases since the beginning of the experiment (data not shown). Cell death (in percentage leaf area) highly correlated (r = 0.92) with the cumulated ethylene dose emitted until the sampling date (Fig. 5). Cumulative ethylene emission dose higher than approximately 1 μmol g⁻¹ FW led to an exponential increase in the cell death of beech leaves.

**Requirement of ethylene for cell death induction in beech seedlings**

To inhibit enzyme biosynthesis in the leaves, ozone-exposed plants (2 × ambient) were treated with the ACC synthase inhibitor aminoethoxyvinyl glycine (AVG) at the times indicated in Fig. 6a (triangles; experiment 3). Inhibition of ACS activity led to 70–90% reduction of ethylene emission throughout the whole experimental period. Starting in early July, cell death was monitored in the ozone-treated plants without AVG reaching approx. 15% leaf area. On the other hand, AVG treatment led to markedly delayed and reduced cell death (3% maximum at the end of September; Fig. 6b). ACC application (1 mM) increased...
ozone-induced cell death by a factor of 3 (data not shown).

Previous results with tomato showed that ozone exposure rapidly and transiently increased the transcript levels of two isoforms of ACC synthase, LE-ACS6 and LE-ACS2, after 1 and 5 h, respectively (Moeder et al. 2002). We cloned genes for ACC synthase (FS-ACS1, FS-ACS2), ACC oxidase (FS-ACO1), ethylene receptor (FS-ETR1) and a putative ethylene-dependent transcription factor (FS-ETRF1; Wang, Li & Ecker 2002) from beech, and determined their long-term expression by quantitative real time PCR. FS-ACS2 was up-regulated after 2 d of ozone exposure and continued to increase (approximately 180-fold gene expression in comparison to pollutant-free air controls) until 15 d of exposure (Fig. 7a). In addition, ozone treatment for 2 d led to an approximately six-fold induction of FS-ACO1 which persisted up to 40 d (Fig. 7a). On the other hand, transcript abundance of FS-rbcS (Fig. 7a) and other genes (not shown) was not altered by ozone.

**Field experiments with adult beech trees**

The above results and those of earlier experiments on ozone responses of trees were altogether obtained with seedlings under controlled chamber conditions. We therefore asked whether ozone exposure of adult beech trees grown in the field also leads to enhanced ethylene synthesis and cell death in the leaves. To this end, five 60-year-old beech trees were continuously exposed throughout 1.5 growing seasons to elevated (2 ×) ozone at the Kranzberg free-air exposure site (Nunn et al. 2002; Werner & Fabian 2002) while another five trees were examined under ambient (1 ×) ozone as a control (Fig. 8a). The threshold O₃...
level for the calculation of the accumulated exposure to ozone above a threshold of 40 nL L\(^{-1}\) (AOT40; Matyssek et al. 2004) is also given in Fig. 8a. AOT40 values were comparable in both growing seasons, for 1× ozone 15 ppm h (2000, 2001), for 2× ozone 62 (2000) and 71 ppm h. Leaves from the shade and sun crown were analysed in parallel. As depicted in Fig. 8b, the levels of conjugated ACC were significantly (\(P < 0.001\)) elevated in the shade leaves and tended to be higher under 2× ambient ozone during both growing seasons (\(P = 0.110\) and \(P = 0.097\) in 2000 and 2001, respectively). Leaves from the sun crown did not show major responses. In contrast, the levels of the diamine putrescine (Fig. 8c) as well as the triamine spermidine (not shown) showed a mirror-like behaviour with higher (\(P < 0.001\)) levels in sun leaves in comparison with leaves of the shade crown. Only minor symptoms of ozone-induced cell death were evident under 2× ambient ozone (Fig. 2e, f and M. Günthardt-Goerg, personal communication).

Treatment of twigs from adult beech trees with the ethylene precursor ACC led to increased cell death and, in particular, leaf loss in crowns exposed to 2× ozone (Fig. 2g, h and Fig. 9). Leaves from three control twigs (2× ozone, no ACC treatment) showed markedly lower cell death development and leaf loss than leaves from three ACC-treated twigs (Fig. 9). ACC was transformed into ethylene under the experimental conditions as ethylene concentrations in the Teflon bags of the ACC treatments were 60% higher than those in the controls. In addition, ACC treatment for 2 h led to a distinct increase of \(FS-ACS2\) transcripts, whereas other genes were unaffected (Fig. 7b). The results were confirmed in 2001 (data not shown) suggesting that ethylene is involved in the spread of cell death in leaves of adult trees in the field.

**DISCUSSION**

European beech is the most important broad-leaved tree species in Central Europe (Ellenberg 1996), and is considered to be relatively sensitive to the air pollutant ozone (Pearson & Mansfield 1994; Langebartels et al. 1997; Grams et al. 1999; Matyssek & Sandermann 2003). Leonardi & Langebartels (1991) and Pearson & Mansfield (1993, 1994) observed a transient increase in stomatal conductance, a persistent decrease in assimilation, elevated visible symptoms as well as premature leaf fall in response to near-
ambient ozone levels. Typically, cell death in beech leaves is found as small (1–2 mm diameter), brown lesions occurring in the intercostal areas after prolonged (weeks to months) exposure to ozone. It is therefore similar to ozone-induced cell death in, for example, tobacco and tomato which is now widely accepted as a form of genetically defined, programmed cell death (Pennell & Lamb 1997; Van Camp, Van Montagu & Inzé 1998; Overmyer et al. 2000; Langebartels et al. 2002). In particular, ethylene was found to be involved as a positive regulator in the initiation and propagation phases of the ‘oxidative cell death cycle’ (Van Camp et al. 1998; Langebartels & Kangasjärvi 2004). Ethylene production in ozone-treated beech seedlings surged to levels of up to 70-fold basal rates, with induction times being as rapid as in herbaceous plants (Schlagnhauffer et al. 1997; Tuomainen et al. 1997), although displaying markedly higher persistence (Fig. 4). Ethylene emission peaked approximately 10 h after the highest ozone concentration was reached (i.e. between 2000 and 2400 h), and remained high until the following day, which resulted in continuously elevated ethylene levels during recurring ozone exposure.

Previous studies with herbaceous plants have shown that individual members of the biosynthetic enzymes and of the receptor family for ethylene are differentially expressed in response to external stimuli (Barry et al. 1996; Moeder et al. 2002). It was suggested that a biphasic regulation occurred in tomato in response to ozone, with the ACC oxidase LE-ACS6 as a highly rapid and LE-ACS2 as a longer-lasting response. We have analysed the genes for FS-ACS1 and 2 as well as ACC oxidase FS-ACO1 in beech leaves which are 69, 70 or 78%, respectively, identical to the sequences in tomato (Moeder et al. 2002). Regarding other deciduous trees, FS-ACS1 and 2 show 77 or 76% identity with Populus ACS1 (AF518326) and ACS2 (AF518327), and 77 or 78% to Citrus sinensis ACS1 (AJ012551) or ACS2 (AJ012696), respectively. We report here that the FS-ACS2 and FS-ACO1 homologues respond to ozone in seedlings as well as to ACC treatment in adult trees (FS-ACO1). As shown in Figs 4 and 7a, recurring ozone exposure led to the build-up of long-lasting peaks in FS-ACS2 and FS-ACO1 expression as well as ethylene emission despite the diurnal variation of the responses. This behaviour is clearly different to that of herbaceous plants in which a single pulse of ozone leads to biphasic induction of specific isoforms of ACS and ACO, ACC levels and ethylene emission within 1–5 h (Mehlhorn & Wellburn 1987; Langebartels et al. 1991; Moeder et al. 2002). It is interesting to note that short-term induction of BP-ACS1 in birch was found in sensitive as well as tolerant clones whereas only BP-ACS2 induction correlated with
ozone sensitivity (Vahala et al. 2003b). It has been postulated that \textit{LE-ACS2} is ethylene-inducible (Barry, Llop-Tous & Grierson 2000; Moeder et al. 2002) which corresponds to the induction of \textit{FS-ACS2} in ACC-treated beech trees (Fig. 7b). Ozone-induced ethylene in beech may therefore stimulate its own production by a self-amplifying cycle which lasts for days to weeks.

Figure 4 shows that a persistent surge of ethylene production precedes the formation of cell death lesions and leaf loss in beech seedlings. We therefore related the cumulative ethylene dose to the cell death area of beech leaves and found a highly significant non-linear correlation between these two parameters. Timing as well as magnitude of ethylene production correlated with ozone-induced cell

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure8}
\caption{(a) Annual time course of 1 x and 2 x ambient ozone concentrations at Kranzberg Forest (data from Nunn et al. 2002) of the years 2000 and 2001. (b), (c) Contents of conjugated ACC (b) and of the diamine putrescine (c) in leaves from shade and sun crowns of adult beech trees exposed to the 1 x or 2 x ozone regime.}
\end{figure}
death. It is speculated that beech leaves enter the ‘oxidative cell death cycle’ above a certain threshold level of ethylene (here: approximately 1 μmol g⁻¹ fresh weight; Van Camp et al. 1998; Langebartels & Kangasjärvi 2004) and ultimately proceed to cell death and leaf loss. To further investigate the role of ethylene in ozone-induced cell death, beech seedlings, exposed to 2× ozone levels, were treated with the ACC synthase inhibitor AVG. Our previous results had indicated that ozone injury was reduced in tomato when ethylene synthesis was prevented by means of inhibitors of ACC synthase and oxidase (Tuomainen et al. 1997; Moeder et al. 2002). Ethylene was also required to promote cell death at the time of lesion development in sensitive birch and hybrid poplar (Vahala et al. 2003a, b). In a similar manner, inhibition of ACC synthase activity in beech seedlings resulted in a marked reduction in cell death (Fig. 6). It is therefore postulated that ethylene has an active role in the regulation of cell death in European beech. It has, however, to be demonstrated in future experiments that characteristics of ‘programmed cell death’ (Greenberg 1997) are present in ozone-exposed trees.

The above results as well as most data from the literature were obtained under controlled conditions in closed chambers, greenhouses or in open top chambers (Sandermann 1996; Matyssek & Sandermann 2003). In addition, there is increasing awareness that juvenile trees may differ in their physiology, and thus in ozone response, from mature individuals (Matyssek & Sandermann 2003). It was therefore of interest to investigate the role of ethylene in ozone sensitivity under field conditions in adult trees. To this end, ethylene production, cell death and leaf loss were recorded under 1× and 2× ambient ozone in a canopy O₃ exposure experiment at Kranzberg Forest (Nunn et al. 2002; Werner & Fabian 2002). It was shown that the levels of conjugated ACC were elevated in shade leaves when treated with 2× ambient ozone. Similar to herbaceous plants (Langebartels et al. 1991), polyamines exhibited a mirror-like behaviour with highest levels in the sun-exposed leaves. Ethylene and polyamines share the same precursor, S-adenosylmethionine (SAM) and mutually inhibit their biosynthesis (Zarembinski & Theologis 1994). Application of polyamines reduced ozone-induced cell death in tobacco and other species (Langebartels et al. 2002; Navakoudis et al. 2003). A metabolic switch towards ethylene or polyamine biosynthesis has therefore been postulated as a major factor in the O₃ sensitivity of plants (Langebartels et al. 1991).

Ambient and twice ambient ozone levels did not lead to significant visible leaf injury in both growing seasons. The AOT40 values under 1× ozone (15 ppm h in years 2000 and 2001) and 2× ozone (62 and 71 ppm h in 2000 or 2001, respectively, all exceeded the ‘critical level’ of 10 ppm h for trees (Fuhrer et al. 1997; Karlsson et al. 2004). It has to be pointed out, however, that the ozone levels in the 2× ambient regime were confined to maximum 150 nL L⁻¹ in the field, not in the chamber treatments. It may be speculated that 2× ozone without this limit would, due to plant responses to high ozone levels, also lead to visible injury in the field. Pharmacological experiments with added ACC to ozone-exposed twigs revealed that cell death and more so leaf loss were markedly elevated in comparison to the ACC-free control treatment. Therefore, our results from both ontogenetic stages, seedlings and adult trees in the field, suggest that ethylene has a regulatory role in cell death and subsequent leaf loss in broad-leaved beech. It is tempting to speculate that a certain threshold of ethylene has to be generated that subsequently drives cells into the oxidative cell death pathway (cf. Van Camp et al. 1998; Langebartels & Kangasjärvi 2004). From this point, ethylene may either promote leaf loss independently, or a certain degree of cell death is necessary for leaf loss to be activated. Taken together, ozone, once taken up by the stomata, initiates an amplifying programme, the oxidative cell death cycle, with ethylene as a key player which leads to cell death and accelerated leaf fall. This mechanism will be incorporated into new, ecologically meaningful critical levels for ozone and quantitative risk assessment of the impact of ozone on trees.
ACKNOWLEDGMENTS

We thank H.-D. Payer as well as P. Fabian and H. Werner for their co-operation in the closed chamber experiments and at Kranzberg Forest, respectively. The expertise of M.S. Günthardt-Goerg, Ozone Validation Centre, Birmensdorf, CH, in assessing ozone symptoms in the field is highly appreciated. We also thank L. Gossel and R. Ludwig for skilful technical assistance and R. Kreitmeyer and B. Gross for growing of the plants. D. Strube and W. Kratlz are acknowledged for their help in ozone exposure and closed chamber performance, respectively. We also thank Thomas Feuerbach, Peter Kuba, Nick Hofmann and Anton Knöttig for their assistance in free-air exposure of adult trees and other technical support at Kranzberg Forest. This study was supported by the Bayerisches Staatsministerium für Umwelt, Gesundheit und Verbraucherschutz and the Deutsche Forschungsgemeinschaft through SFB 607 ‘Growth and Parasite Defence – Competition for Resources in Economic Plants from Agronomy and Forestry’.

REFERENCES


Received 8 September 2004; received in revised form 5 December 2004; accepted for publication 19 December 2004