

Mycorrhizosphere Responsiveness to Atmospheric Ozone and Inoculation with *Phytophthora citricola* in a Phytotron Experiment with Spruce/Beech Mixed Cultures

K. Pritsch¹, G. Luedemann², R. Matyssek², A. Hartmann³, M. Schloter³, H. Scherb⁴, and T. E. E. Grams²

¹ Chair of Soil Ecology, Technische Universität München, Ingolstädter Landstraße 1, 85758 Oberschleißheim, Germany

² Ecophysiology of Plants, Technische Universität München, Am Hochanger 13, 85354 Freising, Germany

³ GSF – National Research Center for Environment and Health, Institute of Soil Ecology, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany

⁴ GSF – National Research Center for Environment and Health, Institute of Biomathematics and Biometry, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany

Received: May 9, 2005; Accepted: October 5, 2005

Abstract: The aim was to analyze functional changes in the mycorrhizosphere (MR) of juvenile spruce and beech grown in a mixture under ambient and twice ambient ozone and inoculated with the root pathogen *Phytophthora citricola*. The phytotron experiment was performed over two vegetation periods, adding the pathogen at the end of the first growing season. Root biomass data suggest that the combined treatment affected spruce more than beech and that the negative influence of ozone on stress tolerance against the root pathogen *P. citricola* was greater for spruce than for beech. In contrast, beech was more affected when the pathogen was the sole stressor. The functional soil parameter chosen for studies of MR soil samples was activity of extracellular enzymes. After the first year of ozone exposure, MR soil samples of both species showed increased activity of almost all measured enzymes (acid phosphatase, chitinase, β -glucosidase, cellobiohydrolase) in the O₃ treatment. Species-specific differences were observed, with a stronger effect of *P. citricola* on beech MR and a stronger ozone effect on spruce MR. In the second year, the effects of the combined treatment (ozone and *P. citricola*) were a significant increase in the activity of most enzymes (except cellobiohydrolase) for both tree species. The results indicated that responsiveness of MR soils towards ozone and *P. citricola* was related to the severity of infection of the plants and the reduction of belowground biomass, suggesting a strong, direct influence of plant stress on MR soil enzyme activity. Additional research is needed using different species and combined stresses to determine the broader ecological relevance of shifts in rhizosphere enzymes.

Key words: O₃, *Fagus sylvatica*, *Picea abies*, rhizosphere, soil enzyme activity, biomass, root rot pathogen.

Introduction

Atmospheric ozone, one of the potentially most phytotoxic air pollutants, has increased in recent decades by an average of 1–2% per year (Stockwell et al., 1997). Moreover, ozone levels are expected to remain high in the forthcoming decades (Fabian, 2002). In forest ecosystems, ozone is known to cause chronic stress to plants, with unknown consequences for individual tree fitness in the long term (Matyssek and Innes, 1999). Beyond direct injuries to plants by ozone, one major concern is that ozone may increase the predisposition for diseases or may lower competitiveness of species (Matyssek and Innes, 1999; Matyssek and Sandermann, 2003). Contrary to this assumption, it has been hypothesized that plants growing under ozone stress produce more stress-related compounds than unstressed plants and therefore may develop stimulated defences against a possible pathogen (cf. Matyssek and Sandermann, 2003). However, in the long term, ozone may weaken resistance to parasite attack by “over-stretching” plant defence mechanisms (Matyssek and Innes, 1999).

Up to now, very few ecologically relevant experiments have considered the effects of ozone in combination with biotic stress such as pathogens. Recently, the root rot pathogen *Phytophthora citricola* Sawada has been studied in German forests (Jung, 2004), where it is responsible for severe damage to adult European beech trees (*Fagus sylvatica* L.). *P. citricola* can also cause seedling death of European beech in bare root nurseries (Werres, 1995), but appears to have a broader host range, at least under experimental conditions, where Norway spruce seedlings (*Picea abies* [L.] Karst.) have also been found to be susceptible to this pathogen (Nechwatal and Oßwald, 2001).

Despite numerous studies on the role of ozone on plant growth and development, there is limited understanding regarding the possible effects of ozone on soil processes (Andersen, 2003). In soils, ozone is rapidly inactivated and does not directly act as a noxious oxidative substance. More likely, ozone alters carbon flux from plants to the soil through altered rhizodeposition (exudation, root, and hyphal turnover) and changes in leaf litter quantity and quality, which affects interactions between roots, rhizosphere organisms, and decomposer communities (Andersen, 2003). Soil enzyme activities that are in-

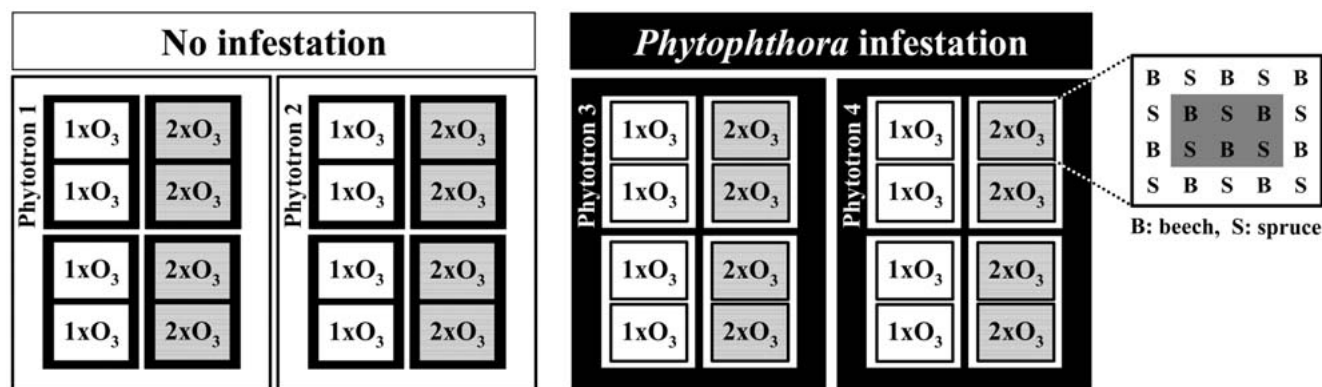


Fig. 1 Experimental set-up in the phytotrons of the GSF National Research Center for Environment and Health. Each phytotron comprised four Plexiglas sub-chambers (for individual O₃ fumigation) with (Phytotron 3 and 4) or without *Phytophthora* infestation (Phytotron 1 and 2). Two planting containers with mixed beech/spruce cultures were placed

into each of the four Plexiglas sub-chambers per phytotron. The experimental set-up in chambers 1 and 3 was reproduced in chambers 2 and 4. Spacing of beech (B) and spruce (S) seedlings in a planting container is shown to the right. The position of the studied trees (six central trees per container) is highlighted.

involved in nutrient turnover and mobilization may be sensitive to changes in soil chemistry (Marx et al., 2001) that are caused by altered exudate and litter composition under ozone stress.

The present study focused on belowground responses of juvenile European beech and Norway spruce to ozone, to the pathogen *P. citricola*, and to the combination of these two stresses. Field ozone concentrations were simulated in a phytotron experiment with beech and spruce seedlings growing in a mixture with or without the root pathogen *P. citricola* as an additional stressor. We focused on root biomass as well as the activity of four enzymes relevant in nutrient turnover in acidic forest soils (phosphatase, exo- and endocellulase, chitinase) and putative stress reactions (chitinase). Based on the assumption that plant reactions influence processes in the (mycor)rhizosphere and that ozone stimulates similar defence mechanisms in plants as those caused by a pathogen, the hypotheses were tested that: a) mycorrhizosphere processes are similarly influenced by ozone and a pathogen, and b) the addition of a pathogen to ozone-treated plants will not further influence mycorrhizosphere processes due to stimulated plant defence mechanisms.

Materials and Methods

Soil chemical and physical parameters

In spring 2001, natural soil (dystric cambisol) was taken from the mixed spruce/beech stand "Höglwald" (Kreutzer and Weiss, 1998) near Augsburg, Germany, sieved (<2 cm) and filled into containers (0.7 × 0.4 × 0.3 m). The soil for the present study was a mixture of A (about 5%) and B (about 95%) horizons at a pH > 4.5, which has been previously tested to ensure good infection potential for *P. citricola* (F. Fleischmann, pers. comm.).

At each harvest, every soil sample (see below) was sieved and the fraction <2 mm was used for further analysis. pH was determined from three subsamples of each container, each subsample representing 4 g of fresh soil in 10 ml of distilled H₂O. Total C and N analyses were carried out in triplicate from air-

dried and ball-milled soil samples. Portions of 70 mg (three portions for each container) were analyzed using an ion ratio mass spectrometer (Na 1500 Series 2, Carlo Erba Instruments, USA).

At the first harvest, 6 weeks after inoculation with *P. citricola*, pH (H₂O) on average was 4.8 ± 0.3 . The C and N contents were $0.854\% \pm 0.20$ (C) and $0.051\% \pm 0.010$ (N) of dry weight (dw), respectively, resulting in a C:N ratio of 16.6 ± 1.7 . Differences between treatments were not detectable. One year later, these parameters were only slightly different: pH 4.6 ± 0.1 , C content $0.98\% \pm 0.22$, N content $0.06\% \pm 0.01$, and a C:N ratio of 15.7 ± 1.0 . This increase of C in the rhizosphere soil may indicate an increase in organic matter, probably derived from decaying dead roots.

Experimental set-up

A total of 32 containers were planted with 20 plants each (10 one-year-old seedlings of European beech (*Fagus sylvatica* L.) and 10 two-year-old seedlings of Norway spruce (*Picea abies* [L.] Karst.), arranged in rows of 4 × 5 individuals in an alternating pattern (Fig. 1). After the first growing season in a climate controlled greenhouse (2001) with purified air, the containers were transferred into four walk-in phytotrons (size ca. 2.8 × 3.4 m) (for details on the phytotrons see Payer et al., 1993, and Thiel et al., 1996). Each phytotron contained four ventilated Plexiglas sub-chambers (size: ca. 0.8 × 1.1 × 1.0 m), with each Plexiglas sub-chamber offering space for two containers; resulting in a total of 32 containers in 16 Plexiglas sub-chambers in 4 phytotrons (Fig. 1). The planted containers were kept under naturally varying climate conditions for the following two growing seasons (2002, 2003). Hourly temperature, irradiance, and relative humidity in the phytotrons were based on measurements from the study site "Kranzberg Forest" for the growing seasons 1998 and 1999 (Nunn et al., 2002). Similarly, O₃ concentrations followed the same natural climate at ambient or elevated O₃ concentrations (i.e., twice ambient O₃ concentrations; restricted to <150 nl l⁻¹), with two Plexiglas sub-chambers in one phytotron receiving ambient and the other two chambers receiving twice ambient O₃ (Fig. 1). According to Nunn et al. (2002), the simulated climate conditions

represent typical temperature, moisture, and ozone levels for the vegetation period in southern Germany. Monthly means of climate conditions within the phytotrons (day and night temperatures, relative humidity, CO₂ concentrations, and O₃ concentrations) were comparable in both years and are presented in detail by Luedemann et al. (2005). The day/night O₃ concentrations were 21.3–43.4/12.1–24.1 nl l⁻¹ in the ambient and 37.7–76.0/21.2–43.0 nl l⁻¹ in the 2 × ambient treatment (May to October 2002). The corresponding values for May to September 2003 were 31.0–37.7/15.3–23.9 nl l⁻¹ in the ambient and 61.8–75/27.1–45.7 nl l⁻¹ in the 2 × ambient O₃ treatment. The cumulative AOT40 values were 11.6 μl l⁻¹ h in the ambient and 61.4 μl l⁻¹ h in the 2 × ambient O₃ treatment (May–October 2002), and 12.2 in the ambient and 77.1 μl l⁻¹ h 2 × ambient treatment (May–September 2003). Three tensiometers (Model T5, UMS, Munich, Germany) per container continuously monitored soil moisture at a depth of 7 cm and were set to trigger irrigation with de-ionized water whenever soil water tension reached 400 hPa. Double strength Hoagland solution (Hoagland and Arnon, 1950) was added to maintain nutrient levels similar to those found in natural soils of Bavarian forests (cf. Kreuzer and Weiss, 1998). During the winter months of 2001/2002 and 2002/2003, plants were placed outdoors to receive a frost period. At the end of July 2002, plants in two of the four phytotrons were infested with the root pathogen *P. citricola* following the method of Fleischmann et al. (2002). The resulting four O₃/*P. citricola* regimes in each phytotron were: (1) ambient O₃/not inoculated with *P. citricola* hereafter referred to as “control”, (2) elevated O₃/not inoculated with *P. citricola* = “+O3”, (3) ambient O₃/inoculated with *P. citricola* = “+P”, and (4) elevated O₃/inoculated with *P. citricola* = “+O3+P”.

Harvests

Four of the containers were used for other experimental purposes (see Luedemann et al., this issue) which are not part of this study. The remaining 28 containers were harvested at two time points. At each harvest, containers were removed from the phytotrons and cut open with an angle grinder. To minimize potential edge effects, all parameters were determined from each of the 6 central plants of each container. The first harvest was performed in September 2002, at the end of the first growing season under ozone treatment, and 6 wks after inoculation with *P. citricola*. Nine plants per species and treatment were studied at the first harvest. The second harvest was performed one year later, in September 2003. Nine plants per species in the control and +O3 treatment, and 15 plants of each tree species in the +P and +O3+P treatments were studied at the second harvest. The root system of all trees was freed from soil by gentle manual agitation. Rhizosphere soil of each individual plant was collected in plastic bags by shaking off soil clumps (diam. < 1 cm) still adhering to the fine roots. These soil samples, referred to as “mycorrhizosphere soil” samples, were immediately placed in a Styrofoam box on ice and kept at 4 °C until further analysis. Biomass of total plant, small (2–5 mm) and fine roots (< 2 mm) were assessed as dry mass after drying (65 °C) to constant weight.

The successful infestation of *P. citricola* was tested by a specific real-time quantitative PCR method which, together with the detailed results, is described by Luedemann et al. (2005).

Soil enzyme activities

Potential enzyme activities of each mycorrhizosphere soil sample were determined within 4 weeks after sampling. Each sample was sieved (< 2 mm) and 3 portions each of 400 mg were weighed into 50 ml centrifuge tubes (Falcon, Sarstedt, Germany). After adding 40 ml of sterile distilled water and vigorous shaking by hand for 10 s, suspensions were shaken on an overhead shaker for 15 min at room temperature and at maximum speed to ensure thorough mixing. After ultrasonification for 3 min in an ice bath, the soil suspensions were filtered through a 90-μm nylon mesh to remove coarse particles (sand, plant residues, etc.). Although some of the enzyme activities may have been lost by filtration, the highest enzyme activities were found in the smaller particle size fractions < 63 μm, at least in grassland soils (Marx et al., 2005). The filtrates were immediately used for enzyme assays.

The enzyme assays were prepared in black microplates using 4-methylumbelliferone (MU)-labelled enzyme substrates. The four enzyme substrates/corresponding enzymes were MU-phosphate (MU-P)/acid phosphatase (EC 3.1.3.2); MU-β-1,4-glucopyranoside (MU-G)/β-glucosidase (EC 3.2.1.21); MU-cellobiohydrofurane (MU-C)/cellobiohydrolase (EC 3.2.1.91), and MU-β-1,4-N-acetylglucosaminide (MU-NAG)/chitinase (EC 3.2.1.14). All chemicals were derived from Sigma-Aldrich Chemicals (Germany) and solutions were prepared as previously described (Pritsch et al., 2004). In pre-experiments, the required incubation time and the substrate saturation concentration for each substrate was determined with concentrations of 100, 200, 300, 400, 500, 600, 700, 800 μM in the incubation wells. From these results (data not shown), the optimal substrate concentrations in the incubation mix and the required incubation times were: MU-P 800 μM 30 min, MU-G 400 μM 3 h, MU-NAG 500 μM 3 h, MU-C 200 μM 20 h. The incubation mix contained 50 μl each of soil suspension, substrate, and sterile distilled water. Plates were incubated on a microplate shaker under continuous gentle shaking at 21 °C in the dark. Controls for autofluorescence of the substrate contained 100 μl of sterile distilled water and 50 μl of substrate. The reaction was stopped using a mixture of ethanol: Tris 2.5 M, pH 10–11 (3/1 v/v). Prior to fluorescence measurements, microplates were centrifuged for 5 min at 750 × g.

Calibration curves were included in every series of enzyme measurements. The calibration wells contained 50 μl each of soil suspension, sterile distilled water and calibration solutions (0, 100, 200, 300, 400, 500 pmol MU in 50 μl). Fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 450 nm, slit widths of 5 nm, with a Cary Eclipse Fluorescence Spectrophotometer with a microplate reader (Varian, Australia).

From the calibration curves, the concentration of released MU was calculated. Enzyme activities were expressed as MU release in nmol per g soil dry weight and hour (nmol g⁻¹ h⁻¹).

Statistical analysis

Data from enzyme activity measurements were first analyzed for differences between the specific enzyme activities using one-way ANOVA. For pairwise comparison of means, Duncan's multiple range test was applied.

Table 1 Root and total plant biomass [g dw] of juvenile beech and spruce in September 2002 and September 2003, after one or two seasons of ozone treatment and 6 or 58 wks after *P. citricola* inoculation. Treatments: control, +O₃, +P, +O₃+P; n = 6 seedlings in each treatment in 2002; n = 9 seedlings in the treatments control, +O₃, n = 15 in the treatments +P, +P+O₃ in 2003. SE, standard error

Treatment	September 2002				September 2003			
	Root biomass		Total biomass		Root biomass		Total biomass	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Beech								
Control	6.20	0.90	10.14	1.68	13.73	2.64	27.18	5.37
+O ₃	6.07	1.78	10.50	3.18	5.73	1.06	11.71	2.35
+P	5.81	0.61	10.49	1.07	8.74	1.70	16.71	4.02
+O ₃ +P	5.76	0.92	10.92	2.05	8.29	1.74	17.52	4.06
Spruce								
Control	6.44	0.30	18.51	0.86	8.50	1.03	30.28	3.63
+O ₃	5.95	0.38	19.85	2.24	9.65	0.78	32.22	2.23
+P	5.61	0.68	16.85	2.08	10.19	0.72	34.82	2.02
+O ₃ +P	7.79	0.93	24.70	3.43	6.23	0.87	22.74	2.73

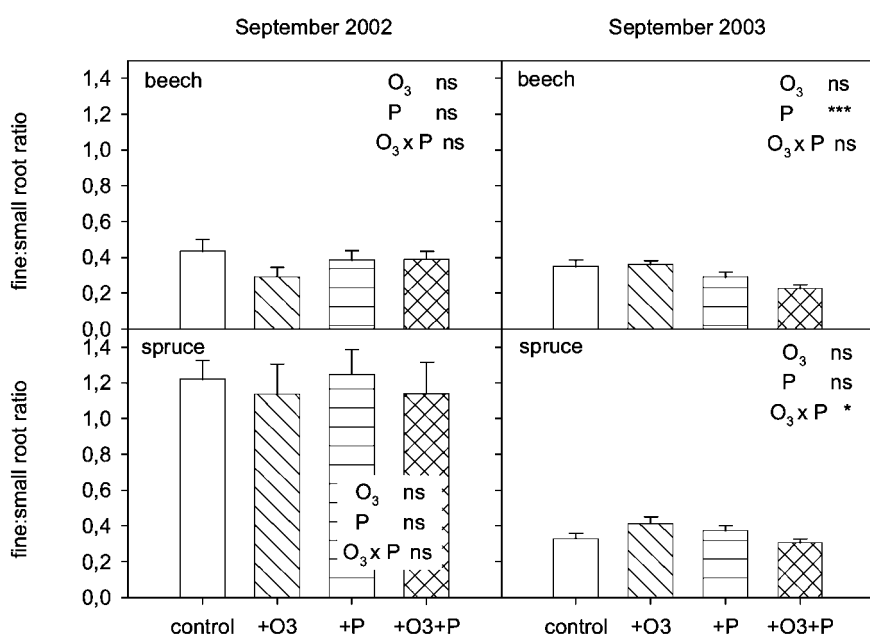


Fig. 2 Fine:small root biomass ratio of beech (top row) and spruce (bottom row) in 2002 after one season of ozone treatment and 6 wks after *P. citricola* inoculation (left column) and in 2003 after two seasons of ozone treatment and 58 wks after *P. citricola* inoculation (right column). Treatments: control, +O₃, +P, +O₃+P; 2002: n = 6 seedlings in each treatment. 2003: n = 9 seedlings in the treatments control, +O₃, n = 15 in the treatments +P, +P+O₃. Error bars indicate standard error, the level of significance for 2-way ANOVA is given in each graph, ns not significant ($p > 0.05$), * $p \leq 0.05$, *** $p < 0.001$.

To explain the effects of treatments and interactions of the two stresses imposed on biomass and enzyme activities, a two-way ANOVA was performed for each species separately, including ozone and *P. citricola* as single factors. For enzyme activities, additional statistical analyses were performed, including the species as third factor. Statistical significance was set at $p < 0.05$ if not mentioned otherwise. Statistical analysis was performed using SPSS 12.0 for Windows (SPSS, USA).

Results

Plant biomass

In September 2002, after one growing season under different ozone regimes and six weeks after inoculation with *P. citricola*, root and total biomass of beech and spruce did not dif-

fer substantially between treatments (Table 1) and no significant treatment effects were observed (statistical data not shown). Spruce generally had greater total biomass and lower root:shoot ratios than beech, which is a species-specific phenomenon of saplings at this age, independent of treatments or competition. Fine to small root ratios differed between beech and spruce, because of the different root architecture in saplings of these two species, but there were no significant effects of treatments on fine:small root biomass ratios after one growing season (Fig. 2).

One year later, in September 2003, root and total biomass of beech decreased in all treatments compared with the control (Table 1), although only the O₃ effect on root biomass was statistically significant ($p < 0.033$). The fine:small root ratio was significantly lower in the +P treatment (Fig. 2) and even low-

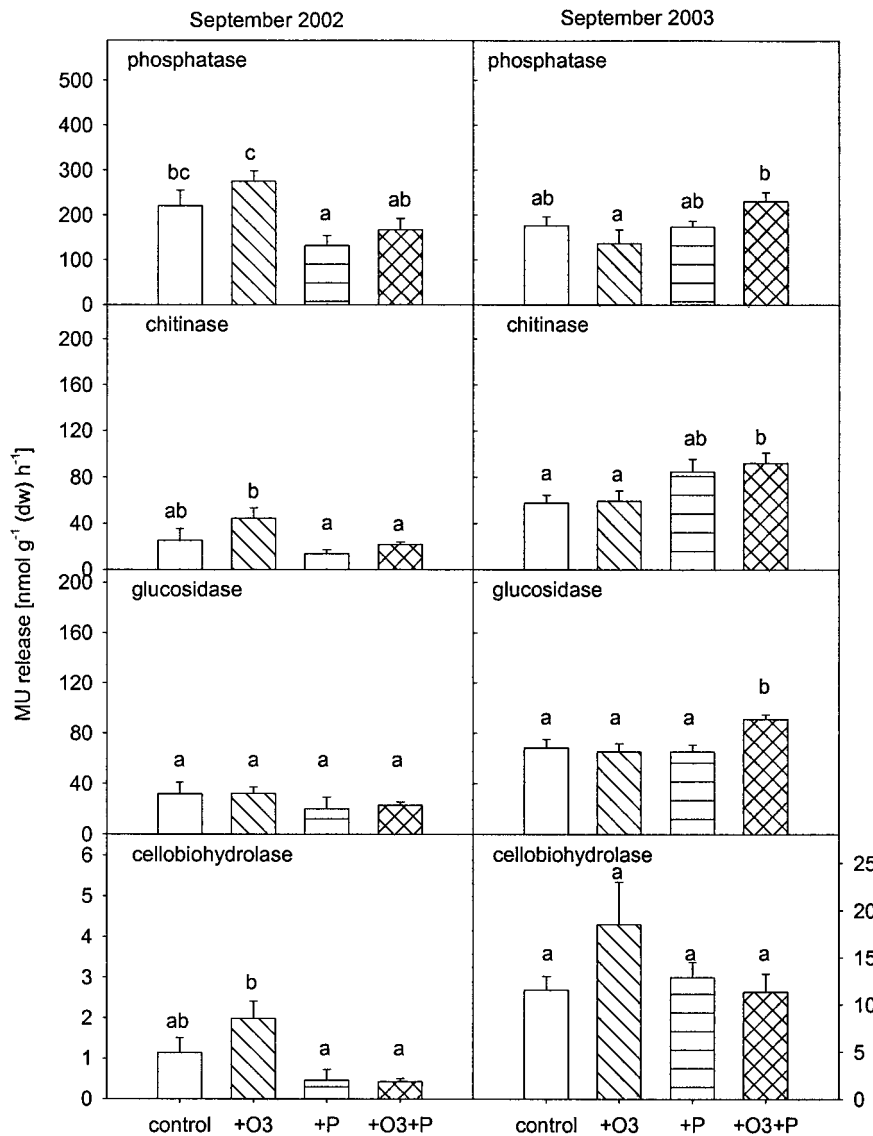


Fig. 3 Enzyme activities of beech mycorrhizosphere soil samples in 2002 and 2003, after one or two seasons of ozone treatment and 6 wks or 13 months after *P. citricola* inoculation. Treatments: control, +O₃, +P, +O₃+P; 2002: n = 6 soil samples; 2003: n = 9 soil samples in the treatments control, +O₃, n = 15 in the treatments +P, +P+O₃ (3 replicate samples for each of 6 seedlings in each treatment). Error bars indicate standard error, different letters above columns indicate statistically significant differences (one-way ANOVA, $p \leq 0.05$).

er in the combined treatment +O₃+P, while the interaction (O₃ × P) was not statistically significant.

In contrast, root and total biomass of spruce was not altered in the +O₃ and the +P treatment, but there was a significant interaction (+O₃ × P) for root biomass ($p < 0.006$) and total biomass ($p < 0.014$) caused by a reduction in both biomass parameters (Table 1) and among roots, especially the fine root biomass. This strong decrease in fine root biomass is also reflected by a significant O₃ × P effect on the fine:small root biomass ratio (Fig. 2).

Soil enzymatic activities

In September 2002, enzyme activity patterns were similar for both tree species: 2 × ozone increased, and *P. citricola* decreased enzyme activities, and in the combined treatment similar or lower activities were observed compared with the control (Figs. 3, 4). This interpretation is supported by the output of the statistical model integrating the species as third var-

iable, which showed that there was a species-specific effect only on cellobiohydrolase activity, independent of the treatment. The results also revealed that there were no significant species interactions with ozone, pathogen or the combined treatment on any of the enzymes after the first year of ozone treatment and 6 wks after *P. citricola* inoculation (Table 3). Rather, there were significant effects of O₃ (pho, chi), *P. citricola* (pho, chi, cel) and a significant interaction of O₃ × P (cel) independent of the species, suggesting a general response of both species in the first year (Table 3). However, when the species were analysed separately under a model integrating O₃, P, and the interaction O₃ × P, there were also differences in the responsiveness of MR soils from the two species. Beech MR soils showed no significant O₃ or combined O₃ × P effects (Table 2), while there was a significant *P. citricola* effect (pho, chi, cel). In contrast, MR soils from spruce showed significant O₃ effects (pho, chi, β-glu) and significant *P. citricola* effects (pho, cel) but an interaction (O₃ × P) only for phosphatase (Table 2).

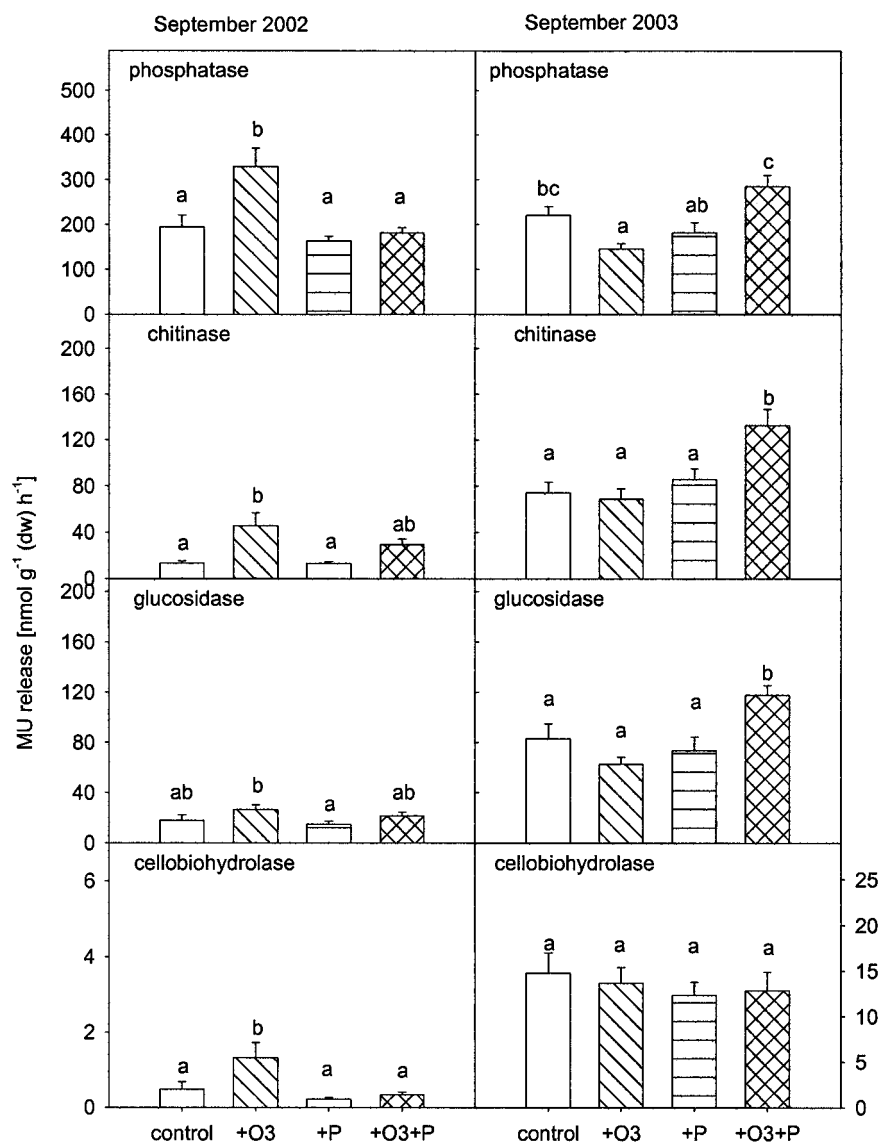


Fig. 4 Enzyme activities of spruce mycorrhizosphere soil samples in 2002 and 2003, after one or two seasons of ozone treatment and 6 wks or 13 months after *P. citricola* inoculation. Treatments: control, +O₃, +P, +O₃+P; 2002: n = 6 soil samples; 2003: n = 9 soil samples in the treatments control, +O₃, n = 15 in the treatments +P, +P+O₃ (3 replicate samples for each of 6 seedlings in each treatment). Error bars indicate standard error, different letters above columns indicate statistically significant differences (one-way ANOVA, $p \leq 0.05$).

One year later, in September 2003, enzyme activity patterns had changed compared with 2002 and eventually differences between the MR soils of both species became more distinct (Figs. 3, 4). The most evident result is a significant increase in enzyme activities (pho, chi, β -glu) for both species in the combined +O₃+P treatment (Figs. 3, 4). There were no significant effects of species in the ozone treatment alone (O₃ × species), but there was a significant effect in the +P treatment (P × species) on β -glucosidase activity, and in the +O₃+P treatment (O₃ × P × species) on chitinase activity (Table 3). It is evident that both effects are due to an increase in these two enzyme activities in the spruce MR (Fig. 4). In contrast to the first year, species-independent effects, such as a significant ozone effect, was only detected for β -glucosidase (+O₃, Table 3), whereas a significant *P. citricola* effect (P, Table 3) and a significant interaction of ozone and *P. citricola* (O₃ × P, Table 3) were observed for three of the studied enzymes (pho, chi, β -glu).

Analyzing the species separately, the treatment effects on beech MR enzyme activities were similar to the first year for O₃ (only significant for β -glu) and *P. citricola* (significant for pho, chi, β -glu), but – in contrast to 2002 – a combined O₃ × P effect was detected (pho, β -glu) (Table 2). Changes in spruce MR soil activities due to treatments were stronger than in beech. In contrast to the first year, none of the enzyme activities was significantly influenced by the O₃ treatment, but there was a clear *P. citricola* and a strong combined O₃ × P effect (pho, chi, β -glu) (Fig. 4, Table 2).

Discussion

Beech and spruce grown in a mixture responded differently to ozone and pathogen stress. The results on the effects of ozone are in good agreement with a previous study under the same experimental setup but with O₃ and CO₂ treatment, in which beech was less competitive compared with spruce under elevated O₃ (Kozovits et al., 2005 a, b). Biomass of beech was lower after two years of elevated O₃ exposure, while spruce bio-

Table 2 *p* and *F* values of statistical analysis on MR soil enzyme activities including *O*₃ and *P. citricola* (*P*) as factors for analysis of variance. pho, phosphatase; chi, chitinase; β-glu, β-glucosidase; cel, cellobiohydrolase. Significant results (*p* ≤ 0.05) are in bold

Factors	September 2002			September 2003			β-glu			cel		
	pho	chi	<i>P</i>	pho	chi	<i>P</i>	F	F	<i>P</i>	F	F	<i>P</i>
Beech												
<i>O</i> ₃	2.9684	0.1030		0.1859	0.6685		4.119	0.6593		1.1538	0.2987	0.0486
<i>P. citricola</i>	13.8845	0.0017		4.7379	0.0350		4.0827	0.0049		10.2527	0.0056	0.0496
<i>O</i> ₃ × <i>P</i>	0.1344	0.7184		5.5245	0.0234		6.5693	0.7696		2.1360	0.1632	0.0140
Spruce												
<i>O</i> ₃	7.7262	0.0128		0.3797	0.5409		3.0503	0.2372		3.4576	0.0804	0.0877
<i>P. citricola</i>	10.6122	0.0046		4.5245	0.0391		10.0637	0.0267		5.9609	0.0259	0.0028
<i>O</i> ₃ × <i>P</i>	4.5209	0.0484		14.3629	0.0005		4.7842	0.0025		2.0446	0.1709	0.0341

mass was not negatively influenced by elevated *O*₃. In the present study, *P. citricola* decreased root biomass and fine : small root ratios of beech but not of spruce, and infections in beech were 10-fold higher than in spruce (Luedemann et al., 2005). Previous findings from infection experiments with *P. citricola* revealed a 30% dieback of fine roots of juvenile spruce but 90% root dieback in beech (Nechwatal and Oßwald, 2001), demonstrating the higher susceptibility of beech.

In contrast to the single treatments, a combination of *O*₃ and *P. citricola* affected root biomass and fine : small root ratios of spruce more than beech. This is again reflected in the infection rates of spruce, which were double in the combined treatment compared with the +*P* treatment (Luedemann et al., 2005). The advantage of spruce over beech in this competitive setup, as shown for ambient and elevated *O*₃ (Liu et al., 2004; Kozovits et al., 2005a, b), disappeared when the pathogen *P. citricola* was added to *O*₃-treated plants. An increase in susceptibility towards the root-borne pathogen *Heterobasidium annosum* under elevated ozone has also been demonstrated in non-mycorrhizal Scots pine seedlings, whereas mycorrhization with *Hebeloma crustuliniforme* completely prevented this negative effect (Bonello et al., 1993). The degree of mycorrhization of the plant roots in the containers was 80 to 90% through the natural, soil-borne inoculum which, according to morphotyping, mainly consisted of *Tomentella* sp., *Laccaria* sp., *Cenococcum*, and boletoid mycorrhizae (S. Raidl, pers. comm.). The antagonistic behaviour of those mycorrhizal associations towards *P. citricola* was not studied. Using non-sterile, natural soil, non-mycorrhizal control seedlings cannot be grown and therefore the effect of mycorrhization was not part of the present study. *H. annosum* and *P. citricola* use different modes of infection and therefore our results and those from Scots pine (Bonello et al., 1993) may not be directly comparable. However, both pathogens had in common that ozone increased the severity of pathogen–host interaction. Belowground and total biomass of spruce treated with ozone and inoculated with the pathogen *P. citricola* clearly was more affected by the combined treatment than by single treatments. This gave a first indication that hypothesis (b), postulating that *O*₃ would reduce pathogen stress, has been rejected for spruce.

Recently, a highly sensitive fluorescent enzyme detection method has been developed (Marx et al., 2001; Wittmann et al., 2004), that allowed us to analyze very small soil sample volumes, making this method especially useful for MR soil samples. The mycorrhizosphere which is highly influenced by the plant root shows the highest microbial activity compared with other soil habitats (Jones et al., 2004). It is therefore appropriate to focus on this compartment when considering soil processes which may be at the detection limit in bulk soil samples. In the present study, significant alteration of enzyme activity was found in MR soil samples of mixed planted, juvenile spruce and beech plants exposed to ozone and pathogen stress. Changes in MR soil enzyme activities were affected by duration of *O*₃ exposure and inoculation, plant species and type of treatment. Therefore, hypothesis (a), that the treatments similarly affect MR processes, has to be rejected for both spruce and beech.

In contrast to biomass parameters, which were significantly influenced only in the second year of treatment, enzyme activities showed significant changes even in the first year of ozone

Table 3 *p* and *F* values of statistical analysis on MR soil enzyme activities of spruce and beech including species, O₃ and *P. citricola* (*P*) as factors for analysis of variance. Significant results (*p* ≤ 0.05) are in bold

Factors	September 2002				September 2003				cel	F	<i>p</i>					
	pho	chi	β-glu	cel	pho	chi	β-glu	cel								
	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>						
Species	0.9587	0.3344	0.0395	0.8436	2.9905	0.0928	4.2672	0.0468	3.3810	0.0694	2.2823	0.1345	7.2466	0.0085	0.0131	0.9091
O ₃	10.2273	0.0030	14.9850	0.0005	1.4680	0.2340	3.9920	0.0540	0.5573	0.4574	1.3427	0.2497	5.8933	0.0173	0.6101	0.4369
<i>P. citricola</i>	24.3116	0.0000	6.8174	0.0133	3.5401	0.0685	16.4330	0.0003	9.1874	0.0032	13.8504	0.0004	13.7482	0.0004	2.1313	0.1479
O ₃ × <i>P</i>	3.1940	0.0828	1.8831	0.1790	0.0031	0.9558	4.2016	0.0484	19.2954	0.0000	6.1040	0.0154	9.2977	0.0030	1.2665	0.2635
Species × O ₃	0.6576	0.4230	1.2038	0.2803	0.6374	0.4302	0.0745	0.7866	0.0316	0.8593	0.2788	0.5989	0.5156	0.4746	0.9249	0.3388
Species × <i>P</i>	0.0539	0.8178	0.8226	0.3708	0.7257	0.4002	1.1528	0.2907	0.0254	0.8736	0.2448	0.6220	4.0250	0.0479	0.1748	0.6769
Species × O ₃ × <i>P</i>	1.6368	0.2094	0.0578	0.8115	0.0886	0.7678	0.0929	0.7624	1.6691	0.1998	4.2097	0.0432	0.7926	0.3758	2.6872	0.1048

treatment and 6 wks after pathogen inoculation. This indicates that plant stress is rapidly changing the soil compartment in close vicinity to the plant root.

The measured MR enzyme activities reflect a combination of enzymes from different groups of organisms in soils (Burns, 1982). Enzymes of plant origin may be present in the (mycor)-rhizosphere and directly reflect plant reactions. Another source of enzymes are microorganisms in the close vicinity to the roots, which may react to altered soil nutrient composition as influenced by changed root deposits (Andersen, 2003) and therefore these changes indirectly reflect plant reactions. Recently, mycorrhizal roots have been shown to exhibit specific extracellular enzyme activity (Pritsch et al., 2004; Courty et al., 2005), but differentiation of the origin of the enzymes is not yet possible. The measured enzyme activities rather reflect changes in the whole system of an MR, regardless of the source contributing to enzyme activities, but in some cases may point towards the likely main contributors, i.e., plant or soil microorganisms. Phosphatase is an omnipresent extracellular enzyme in forest soils and its relevance is underlined by the fact that over 50% of available phosphorus is organically bound (Attiwill and Adams, 1993). An increase in phosphatase activity is usually correlated with a decrease in available phosphate, and the enzyme can be produced by plants, fungi, and other soil microorganisms (Attiwill and Adams, 1993). Since all treatments received the same amount of fertilization, stimulation of phosphatase in the MR may reflect a higher demand for phosphate required in plant metabolism and, in particular, in all energy-consuming processes, thus pointing to a putatively higher energy demand in stressed plants. An increase in fungal propagules and bacteria exhibiting phosphatase activity was found in the rhizosphere of *Sorghum* (Shafer, 1988) challenged with ozone and acid rain at pH 4. In contrast, no significant effects were found on phosphatase activity in soil samples from experiments with loblolly pine seedlings treated with ozone and acid rain (Reddy et al., 1991, 1995). However, Reddy et al. (1991, 1995) assayed pooled air-dried soil samples of one entire planting pot without differentiating the rhizosphere from the bulk soil, which is not directly comparable to fresh MR samples in the present study.

Chitinases are produced by almost all organisms and are used for many purposes. Soil microorganisms utilize chitin-bound nitrogen derived from fungal hyphae and arthropod exoskeletons. The increase in chitinase in the combined O₃/pathogen treatment could have been derived from the decay of hyphae of mycorrhizal fungi, which declined together with the fine roots. Fungi produce chitinases for growth regulation and in interaction with other fungi and therefore the increase of chitin could also reflect an increase in chitinase activity of mycorrhizal fungi as a reaction to the pathogen *P. citricola*. Chitinases of plants are involved in interactions of roots with fungal symbionts or defence reactions against fungal pathogens (Albrecht et al., 1994). This could be another source for increased chitinase activity although, in the case of the oomycete *P. citricola*, this reaction may not be useful for the plant, since oomycetes do not contain chitin in their cell wall. Furthermore, chitinases are part of the general stress responses of plants, including abiotic stress such as ozone (Kasprzewska, 2003). Considering the strong increase in chitinase in the most stressed plant/treatment combination (e.g., spruce +O₃+P), the most likely

explanation for the increased chitinase activity is a general, unspecific stress response by the plant.

The two cellulose-degrading enzymes strongly increased in the second vegetation period, suggesting a higher potential for the degradation of cellulose, possibly reflecting higher amounts of dead plant roots. This assumption is further supported by the increased C content of the MR soil samples in the second year.

The soil enzymes examined in the present study are known to represent relevant functions in forest ecosystems. In future studies, other relevant enzymes such as esterases, which seem to play a major role in forest soil nutrient cycling (Wittmann et al., 2004), and enzymes in the S and N cycle could be included to examine if other functions in the mycorrhizosphere are affected by O₃ and pathogen stress. The present study represents one step in quantifying the extent of belowground changes in response to ozone and combined effects of other stressors, as strongly suggested by Andersen (2003). The collection of more data on relevant soil functional parameters, such as enzyme activities under stress scenarios in experimental laboratory and field conditions, will help to elucidate the meaning of these biochemical changes in the MR and how they influence soil processes in the long term. Additional research is needed using different species and combined stresses to determine the broader ecological relevance of shifts in rhizosphere enzymes.

Acknowledgements

For scientific, technical, and experimental support we thank the following persons: H.-D. Payer and J. B. Winkler (phyto-trons), F. Fleischmann (*P. citricola* inoculation), F. Buegger (C, N analyses), G. Hufnagel (sample preparation, enzyme assays, soil analyses), P. Kuba, I. Süß, and numerous student workers for help during the sampling periods. The study was carried out by two projects (B5, B9) of SFB607, "Growth and Parasite Defence" (www.sfb607.de) funded by the German Research community (DFG). G. Luedemann is grateful to CAPES, Brasília, Brazil for financial support.

References

- Albrecht, C., Asselin, A., Piché, Y., and Lapeyrie, F. (1994) Chitinase activities are induced in *Eucalyptus globulus* roots by ectomycorrhizal or pathogenic fungi, during early colonization. *Physiologia Plantarum* 91, 104–110.
- Andersen, C. P. (2003) Source-sink balance and carbon allocation below ground in plants exposed to ozone. *New Phytologist* 157, 213–228.
- Attiwill, P. M. and Adams, M. A. (1993) Nutrient cycling in forests. *New Phytologist* 124, 561–582.
- Bonello, P., Heller, W., and Sandermann, H. (1993) Ozone effects on root-disease susceptibility and defence responses in mycorrhizal and non-mycorrhizal seedlings of Scots pine (*Pinus sylvestris* L.). *New Phytologist* 124, 653–663.
- Burns, R. G. (1982) Enzyme activity in soil: location and a possible role in microbial ecology. *Soil, Biology and Biochemistry* 14, 423–427.
- Courty, P. E., Pritsch, K., Schloter, M., Hartmann, A., and Garbaye, J. (2005) Activity profiling of ectomycorrhiza communities in two forest soils using multiple enzymatic tests. *New Phytologist* 167, 309–319.
- Fabian, P. (2002) *Leben im Treibhaus. Unser Klimasystem – und was wir daraus machen.* Berlin: Springer-Verlag, pp.258.
- Fleischmann, F., Schneider, D., Matyssek, R., and Oßwald, W. F. (2002) Investigations on net CO₂ assimilation, transpiration and root growth of *Fagus sylvatica* infested with four different *Phytophthora* species. *Plant Biology* 4, 144–152.
- Hoagland, D. R. and Arnon, D. I. (1950) The water culture method for growing plants without soil. Circular 374. Berkley, CA, USA: California Agricultural Experimental Station.
- Jones, D. L., Hodge, A., and Kuzyakov, Y. (2004) Plant and mycorrhizal regulation of rhizodeposition. *New Phytologist* 163, 459–480.
- Jung, T. (2004) *Phytophthora* schädigt Buchenbestände in ganz Bayern. *LWF Aktuell* 43, 36–37.
- Kasprzewska, A. (2003) Plant chitinases – regulation and function. *Cellular and Molecular Biology Letters* 8, 809–824.
- Kozovits, A. R., Matyssek, R., Blaschke, H., Göttlein, A., and Grams, T. E. E. (2005 a) Competition increasingly dominates the responsiveness of juvenile beech and spruce to elevated CO₂ and/or O₃ concentrations throughout two subsequent growing seasons. *Global Change Biology* 11, 1387–1401.
- Kozovits, A. R., Matyssek, R., Winkler, J. B., Göttlein, A., Blaschke, H., and Grams, T. E. E. (2005 b) Aboveground space sequestration determines competitive success in juvenile beech and spruce trees. *New Phytologist* 167, 181–196.
- Kreutzer, K. and Weiss, T. (1998) The Höglwald field experiments – aims, concept and basic data. *Plant and Soil* 199, 1–10.
- Liu, X., Kozovits, A. R., Grams, T. E. E., Blaschke, H., Rennenberg, H., and Matyssek, R. (2004) Competition modifies effects of enhanced ozone/carbon dioxide concentrations on carbohydrate and biomass accumulation in juvenile Norway spruce and European beech. *Tree Physiology* 24, 1045–1055.
- Luedemann, G., Matyssek, R., Fleischmann, F., and Grams, T. E. E. (2005) Acclimation to ozone affects host/pathogen interaction and competitiveness for nitrogen in juvenile *Fagus sylvatica* and *Picea abies* trees infected with *Phytophthora citricola*. *Plant Biology* 7, 640–649.
- Marx, M. C., Wood, M., and Jarvis, S. C. (2001) A microplate fluorimetric assay for the study of enzyme diversity in soils. *Soil Biology and Biochemistry* 33, 1633–1640.
- Marx, M.-C., Kandeler, E., Wood, M., Wermbter, N., and Jarvis, S. C. (2005) Exploring the enzymatic landscape: distribution and kinetics of hydrolytic enzymes in soil particle-size fractions. *Soil Biology and Biochemistry* 37, 35–48.
- Matyssek, R. and Innes, J. L. (1999) Ozone – a risk factor for trees and forests in Europe? *Water, Air and Soil Pollution* 116, 199–226.
- Matyssek, R. and Sandermann, H. (2003) Impact of ozone on trees: an ecophysiological perspective. In *Progress in Botany* (Esser, K., Lüttge, U., Beyschlag, W., and Hellwig, F., eds.), Berlin: Springer Verlag, pp.349–404.
- Nechwatal, J. and Oßwald, W. (2001) Comparative studies on the fine root status of healthy and declining spruce and beech trees in the Bavarian Alps and occurrence of *Phytophthora* and *Pythium* species. *Forest Pathology* 31, 257–273.
- Nunn, A. J., Reiter, I. M., Häberle, K. H., Werner, H., Langebartels, C., Sandermann, H., Heerd, C., Fabian, P., and Matyssek, R. (2002) "Free-air" ozone canopy fumigation in an old-growth mixed forest: concept and observations in beech. *Phyton – Annales Rei Botanicae* 42, 105–119.
- Payer, H. D., Blodow, P., Köfferlein, M., Lippert, M., Schmolke, W., Seckmeyer, G., Seidlitz, H. K., Strube, D., and Thiel, S. (1993) Controlled environment chambers for experimental studies on plant responses to CO₂ and interactions with pollutants. In *Ecosystems Research Report No. 6: Design and Execution of Experiments on CO₂ Enrichment* (Schulze, E. D. and Mooney, H. A., eds.), Brussels: Commission European Communities, pp.127–145.

- Pritsch, K., Raidl, S., Marksteiner, E., Blaschke, H., Agerer, R., Schloter, M., and Hartmann, A. (2004) A rapid and highly sensitive method for measuring enzyme activities in single mycorrhizal tips using 4-methylumbelliferone labelled fluorogenic substrates in a microplate system. *Journal of Microbiological Methods* 58, 233–241.
- Reddy, G. B., Reinert, R. A., and Eason, G. (1991) Enzymatic changes in the rhizosphere of loblolly pine exposed to ozone and acid rain. *Soil Biology and Biochemistry* 23, 1115–1119.
- Reddy, G. B., Reinert, R. A., and Eason, G. (1995) Loblolly pine needle nutrient and soil enzyme activity as influenced by ozone and acid rain chemistry. *Soil Biology and Biochemistry* 27, 1059–1064.
- Shafer, S. R. (1988) Influence of ozone and simulated acidic rain on microorganisms in the rhizosphere of Sorghum. *Environmental Pollution* 51, 131–152.
- Stockwell, W. R., Kramm, G., Scheel, H.-E., Mohnen, V. A., and Seiler, W. (1997) Ozone formation, destruction and exposure in Europe and the United States. In *Ecological Studies 127: Forest Decline and Ozone. A Comparison of Controlled Chamber and Field Experiments* (Sandermann, H., Wellburn, A. R., and Heath, R. L., eds.), Berlin, Heidelberg, New York: Springer, pp.1–38.
- Thiel, S., Döhning, T., Köfferlein, M., Kosak, A., Martin, P., and Seidlitz, H. K. (1996) A phytotron for plant stress research: how far can artificial lighting compare to natural sunlight? *Journal of Plant Physiology* 148, 456–463.
- Werres, S. (1995) Influence of the *Phytophthora* isolate and the seed source on the development of beech (*Fagus sylvatica*) seedling blight. *European Journal of Forest Pathology* 25, 381–390.
- Wittmann, C., Kahkonen, M. A., Ilvesniemi, H., Kurolo, J., and Salkinoja-Salonen, M. S. (2004) Areal activities and stratification of hydrolytic enzymes involved in the biochemical cycles of carbon, nitrogen, sulphur and phosphorus in podsolized boreal forest soils. *Soil Biology and Biochemistry* 36, 425–433.

K. Pritsch

GSF – National Research Center for Environment and Health
Chair of Soil Ecology
Technische Universität München
85758 Oberschleißheim
Germany
E-mail: pritsch@gsf.de

Editor: H. Rennenberg