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Ozone fumigation (twice ambient) reduces leaf infestation following natural and artificial inoculation by the endophytic fungus *Apiognomonia errabunda* of adult European beech trees

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Chronic sublethal ozone exposure reduces both natural and artificial infestation of beech leaves by the endophytic fungus Apiognomonia errabunda.

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ABSTRACT

In 2006, a controlled infection study was performed in the 'Kranzberger Forst' to address the following questions: (1) Will massive artificial inoculation with *Apiognomonia errabunda* override the previously observed inhibitory effect of chronic ozone? (2) Can biochemical or molecular markers be detected to account for the action of ozone? To this end six adult beech trees were chosen, three ozone fumigated (2× ozone) and three control trees (ambient = 1× ozone). Spore-sprayed branches of sun and shade crown positions of each of the trees, and uninoculated control branches, were enclosed in 100-L plastic bags for one night to facilitate infection initiation. Samples were taken within a five-week period after inoculation. *A. errabunda* infestation levels quantified by real-time PCR increased in leaves that were not fumigated with additional ozone. Cell wall components and ACC (ethylene precursor 1-amino cyclopropane-1-carboxylic acid) increased upon ozone fumigation and may in part lead to the repression of fungal infection.

1. Introduction

Tropospheric ozone is one of the most harmful air pollutants to trees, and it has been broadly studied at the physiological level over the last decades (Fowler et al., 1999; Matyssek and Sandermann, 2003). It has been shown recently that chronic sublethal ozone exposure (twice ambient) reduces infestation by the endophytic fungus *Apiognomonia errabunda* (Rob.) Höhn (anamorph: *Discula umbrinella* [Berk. and Broome] Sutton) in leaves of adult European beech (Bahnweg et al., 2005). The ascomycete *A. errabunda* is a constant companion of European beech detected not only in leaves, but also in buds, twigs, and bark of almost any European beech tree throughout a growing season (Sieber and Hugentobler, 1987; Kowalski and Kehr, 1996) and frequently disease symptoms are absent (Petrini, 1991; Sinclair and Cernauskas, 1996; Stone et al., 2000). In leaves, particularly of the shade crown, *A. errabunda* can cause pronounced symptoms (leaf anthracnose). The extent of infestation, however, strongly depends on climatic conditions. Cool and rainy weather favors spread of the fungus, whereas hot and dry conditions dramatically reduce populations of fungal endophytes (Bahnweg et al., 2005). However, a smaller (but nonetheless significant) reduction of *A. errabunda* populations in shade leaves was observed under chronic twice ambient ozone fumigation (Bahnweg et al., 2005).

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At the experimental site "Kranzberger Forst" (Pretzsch et al., 1998; Werner and Fabian, 2002) adult European beech trees (65 years old, approx. 30 m high) have been subjected to free-air ozone fumigation since May 2000. Long-term twice ambient ozone exposure was achieved for a small number of trees offering a unique opportunity to study the effects of chronic ozone challenge on the interaction of beech trees and their endophytes under natural conditions.

Based on previous results of studies with juvenile beech trees in exposure chambers and greenhouses, we expected to see (1) an induction of defence-related gene transcripts and accumulation of protective compounds upon sublethal ozone exposure leading to (2) a markedly reduced colonisation of the leaves by endophytic *A. errabunda* even after massive artificial inoculation.

2. Materials and methods

2.1. The "Kranzberger Forst" experimental site and ozone treatment

The "Kranzberger Forst" research site is located in Southern Germany near Freising at 48°25′08″N, 11°39′41″E, 485 m a.s.l. (Pretzsch et al., 1998; Werner and Fabian, 2002) in a mixed 65-year-old stand (closed canopy) with about 30 m high European beech (*Fagus sylvatica*) and Norway spruce (*Picea abies*) trees. Scaffolding and a research crane provide access to sun and shade crowns. Starting in May 2000, ten neighbouring individuals (5 beech and 5 spruce trees) were subjected to free-air ozone fumigation within a canopy volume of 2000 m^3 throughout the growing seasons (Werner and Fabian, 2002). Ozone was generated from oxygen-enriched air and fumigated canopy concentrations were adjusted real time to twice ambient conditions. A cut-off at 150 nl l^{-1} was implemented in order to prevent acute ozone injury (Reich, 1987; Nunn et al., 2002). Nearby control trees were exposed to ambient air (1× ozone). The enhanced ozone exposure (2× ozone) was released from a tubing system suspended throughout the canopy.

2.2. A. errabunda cultures and artificial inoculation of adult beech

Axenic cultures of *A. errabunda* were prepared using European beech leaves from the "Kranzberger Forst" experimental site in October 2005 by conventional plating technique. Surface sterilised (1 min 60% ethanol, 1 min 1% NaOCl, 2 min sterile H_2O) leaves were cut into 5 mm strips and plated on 2% malt extract agar. Fungal mycelia were cut from the edge of the outgrowing hyphae and were transferred to new malt agar plates. Identities of new isolates were determined by microscopic observation and were verified by *Apiognomonia*-specific PCR (Bahnweg et al., 2005). Cultures were maintained on oatmeal agar under sterile water at 15 °C. In March 2006, new malt extract agar plates were inoculated, incubated at 22 °C in the dark until about two thirds of the plates were overgrown. Then 6 mm i.d. pieces were cut from the growing edge of the mycelium and transferred to 10 plates of oatmeal agar. These plates were incubated at 22 °C in the dark for 2 weeks (mycelium then covered more than two thirds of the plates) and then placed upside down on a transilluminator for 30 s (UV treatment induced sporulation). Plates were further incubated at a window exposed to the North at 22 °C (room temperature) for 6 weeks until spores were harvested. Immediately before the inoculation (June 28th), spores were harvested by repeatedly pouring 10 ml spore suspension buffer (62.5 mM KH_2PO_4, 5.5 mM glucose, 0.1% Tween 20, pH 6.0) onto the mycelial surface, gently rubbing spores off the plates with a spatula. Spores from six plates were combined and suspended in 1.5 L of the suspension buffer. Spores were counted microscopically and adjusted to 10^5 spores per ml.

At the end of May, six beech trees were chosen, three ozone-fumigated and three control. Four branches per tree were selected and labelled, two in the sun and two in the shade crown. The selected branches were easily accessible by means of the scaffolding and each had at least 50–100 leaves.

June 28th, 2006, which was cool and overcast, was selected for inoculation. In late afternoon (5 p.m.), 100-L plastic waste bags (PVC) were wrapped over the selected branches. Then 100 ml blank suspension buffer without spores was sprayed (manual vaporiser) inside one bag in the shade and another in the sun crown of each of the six selected trees (inoculation controls). This procedure was then repeated with the remaining branches with the spore suspension (inoculated). Care was taken to spray the suspension as evenly as possible inside the bags and to moisten all leaves. The bags were kept closed (tied with strings around the branch) overnight until 8 a.m. of the next morning to maintain high humidity, facilitating conidial infection, and were then removed.

Samples (5–10 leaves = approx. 1–1.5 g fresh weight) were taken from each of the 24 marked branches 2, 5, 9, 15, and 40 d after inoculation. The samples were frozen in liquid nitrogen on the site and stored at −80 °C until processing.

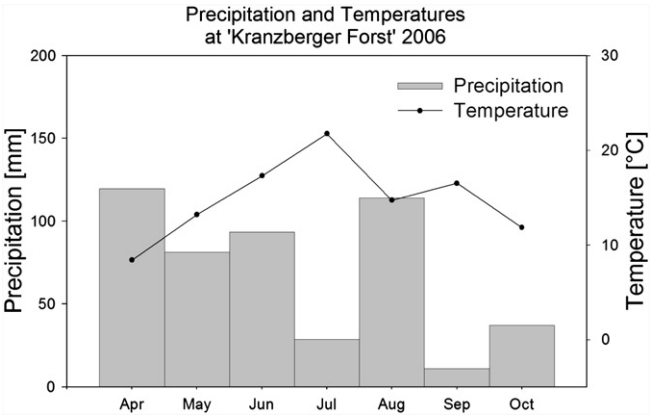

Fig. 1. Precipitation and temperatures measured at "Kranzberger Forst" site in 2006. A mild and moist spring was followed by a warm and dry period in July.

2.3. Fungal quantification, analysis of cell wall components, secondary metabolites, plant hormones, and statistics

DNA extraction from beech leaves, quantification of fungal DNA by quantitative (real-time) PCR using specific primers, analysis of secondary metabolites (soluble phenolics), and of plant hormones [1-amino-cyclopropane-1-carboxylic acid (ACC), salicylic acid (SA)] was performed as described previously (Bahnweg et al., 2005; Nunn et al., 2005). Cellulose determination was adopted from Updegraff (1969). The test was miniaturized using 2-ml Eppendorf screw-cap vials and a starting amount of 100 mg leaf material. The residue of acetic/nitric acid treatment was quantified gravimetrically after lyophilisation. Lignin was determined according to Bruce and West (1989) with 60 mg of finely powdered 1:1 mixtures of leaf tissue and diatomaceous earth. The thioglycolic acid conjugate was dissolved in oxygen-free 0.5 N NaOH and quantified photometrically.

Three of the six investigated trees received ambient ozone while the remaining three trees were exposed to twice ambient ozone. Therefore, the variable "individual tree" is nested in the variable "ozone treatment". Because all other combinations of group levels were complete, a nested ANOVA was used in the model of extracted fungal DNA depending on the factors ozone (nested trees), infection method, sun/shade, and categorical time.

2.4. RNA extraction and analysis of microarrays

Total RNA was extracted from leaf material according to Kiefer et al. (2000); this protocol was applied for tissues containing high amounts of phenolic compounds. The RNA yield and quality were determined by spectral photometry at 230, 260 and 280 nm.

Transcript analysis was performed by using a microarray spotted with 1248 ozone- and 1172 *Phytophthora citricola*-responsive ESTs, obtained via suppressive subtractive hybridisation (Olbrich et al., 2005; Schlink, 2009). In addition 11 ESTs associated with nitrogen metabolism (Stölcken, Freiburg, Germany) and 20 ESTs associated with secondary metabolism were included on the microarray. cDNA was spotted on aldehyde slides (Genetix, Dornach, Germany) using a Microgrid II spotter (Biorobotics, Cambridge, United Kingdom). Reverse transcription, labelling and

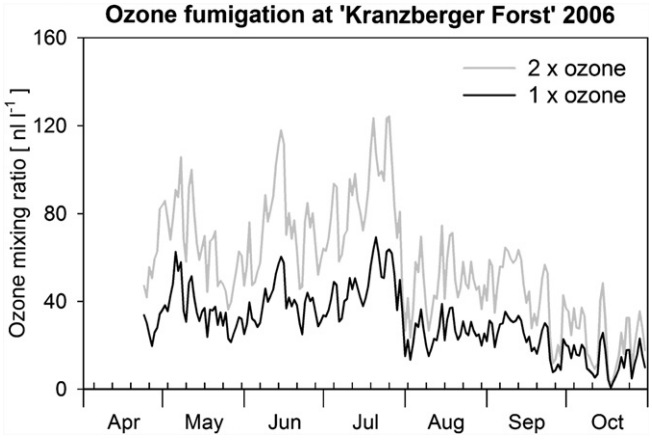

Fig. 2. Ozone regimes (ambient and enhanced) at the "Kranzberger Forst" site in 2006. A predisposing peak in June is followed by elevated ozone in July, mainly due to higher temperatures and sunny weather.