Folia Forestalia Polonica, series A, 2014, Vol. 56 (3), 149–156

ORIGINAL ARTICLE

DOI: 10.2478/ffp-2014-0016

Effect of CO_2 enhancement on beech (*Fagus sylvatica* L.) seedling root rot due to *Phytophthora plurivora* and *Phytophthora cactorum*

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Abstract

Global climate change is associated with higher concentrations of atmospheric carbon dioxide (CO_2). The ongoing changes are likely to have significant, direct or indirect effects on plant diseases caused by many biotic agents such as phytopathogenic fungi. This study results showed that increased CO_2 concentration did not stimulate the growth of 1-year-old beech *Fagus sylvatica* L seedlings but it activated pathogenic *Phytophthora* species (*P. plurivora* and *P. cactorum*) which caused significant reduction in the total number of fine roots as well as their length and area. The results of the greenhouse experiment indicated that pathogens once introduced into soil survived in pot soil, became periodically active (in sufficient water conditions) and were able to damage beech fine roots. However, the trees mortality was not observed during the first year of experiment. DNA analyses performed on soil and beech tissue proved persistence of introduced *Phytophthora* isolates.

Key words

Phytophthora, *Fagus sylvatica,* disease, DNA

Introduction

Phytophthora species have been considered one of the most dangerous plant pathogens in the world. These fungus-like organisms are responsible for the most important plant diseases in many parts of the world. Recently, the large number of *Phytophthora* species causing root damages has become crucial in the protection of European forests (Brasier 1999).

Beech (*Fagus sylvatica* L.) is one of the most economically important tree species in central Europe and its share in forest stands reaches over 50% (Ammer et al. 2005; Kölling et al. 2005; Felbermeier and Mosandl 2006). For many years, beech pathogens i.e. fungi *Nectria* spp. and beech scale *Cryptococcus fagisuga*

Received 18 June 2014 / Accepted 22 July 2014

were considered as the main problem related to bark infection (Felbermeier and Mosandl 2006). This situation has changed in the last decade, since the damage typical of *Phytophthora* root rot was reported in beech stands (Jung 2009). Infections of beech trees by pathogenic *Phytophthora* species have been known in Europe since the 30s of XX century, when the main perpetrators of the damage were species such as *P. syringae* and *P. cambivora* (Day 1938, 1939). Later reports on these pathogenic organisms came from Germany (Jung and Blaschke 1996), where damages by *P. cambivora* and *P. citricola* (nowadays *P. plurivora*) were observed. During the last 10 years the phenomenon associated with beech weakening and decline due to *Phytophthora* pathogens has been observed throughout entire Europe (Balci and Halmschlager 2003; Motta et al. 2003; Cech and Jung 2005; Jung et al. 2005; Belisario et al. 2006; Diana et al. 2006; Brown and Brasier 2007; Denman et al. 2007; Munda et al. 2007; Schmitz et al. 2007). So far, *P. cactorum* has been most frequently isolated from exudates appearing on beech trunks (Belisario et. al. 2006; Cerny et. al. 2009). Fleichmann et al. (2002, 2004) investigated four *Phytophthora* species isolated from declining mature beech trees, with regard to their aggressiveness towards beech seedlings. *Phytophthora plurivora* turned to be the most aggressive pathogen among the tested ones, causing visible fine root loss over two vegetation periods. Total lengths of the fine root systems in the plants tested were strongly reduced when compared to healthy control seedlings.

Changes in the chemical composition of atmosphere, including those of carbon dioxide (CO_2) concentration have direct biological effects on plants. Such effects may directly or indirectly affect the incidence and severity of plant diseases caused by biotic agents (Manning and Tiedemann 1995). According Grobel et al. (2006) ambient CO_2 concentration observed in nature is approximately 400 ppm, but higher CO_2 concentration ranging from 800 to 1000 ppm is beneficial for plant growth.

At the present time, *Phytophthora* species, and in particular *P. cactorum* – recently isolated from infected beech bark and *P. plurivora* – already observed in Polish beech stands, are of high importance (Orlikowski et al. 2004, 2006). An attempt to verify the activity of these harmful organisms in elevated (800 ppm) and ambient (400 ppm) CO_2 concentrations was the purpose of the experiments described in this paper. The results presented may help better prediction of future distribution of pathogens and estimate damages caused to beech stands. The working hypothesis was that under enhanced CO_2 concentration, beech seedlings would grow better but *Phytophthora* species would cause more severe damage.

Material and methods

Preparation of inoculum (*Phytophthora* mix)

Preparation of semiliquid medium

Inoculum containing 250 cm^3 of vermiculite, 20 cm^3 of millet and 175 cm^3 of V8 medium (100 ml of vegetable juice, 2 g of calcium carbonate $CaCO_3$, 900 ml of distilled water) was prepared for the experiment. Liquid V8 medium was sterilized for 15 minutes at 121°C and then mixed with vermiculite and millet in Erlenmeyer flasks (890 cm^3 of medium per each flask). The semiliquid medium was subsequently sterilized for additional 15 minutes at 121°C.

Establishing semiliquid *Phytophthora* cultures

The medium was inoculated with two *Phytophthora* species: *P. cactorum* (isolate IBL 235) and *P. plurivora* (isolate IBL 206) through transferring agar plugs overgrown with 7-day-old mycelium of selected pathogens. *Phytophthora* cultures in semiliquid media were subject to 6-week incubation at 22°C, and then the growth of *Phytophthora* spp. was confirmed through the planting of approximately 0.5 cm^3 of inoculum on solid V8 medium. After the incubation was completed, the inoculum was rinsed with distilled water in order to remove the nutrients from V8 liquid medium.

Design of the experiment and artificial inoculation

A greenhouse facility was divided into 2 gas-tight cabins equipped with a climate control unit. The current outdoor atmospheric conditions (temperature, humidity) were simulated in each greenhouse cabin, which were shaded when the light intensity exceeded 50 klx. Plants were irrigated manually with tap water. CO_2 concentration in the greenhouse was controlled automatically by the computer software as well as the way of maintenance of elevated concentration of carbon diox-

ide which was additionally inserted into the experimental system. To every variants was used 60 1-year-old beech seedlings growing in the greenhouse on sterile growing substrate (mixed soil with perlite, pH 5,5) in the section with enhanced carbon dioxide (concentration: 800 ppm) and to the same number of 1-year-old beech trees in the section with ambient concentration of the gas (400 ppm of CO_2). In both parts of the greenhouse (with ambient and enhanced CO_2) there were the same numbers of plants per treatment: 15 seedlings in the control, and 15 – in the group inoculated by *Phytophthor*a.

The time-frame of the study was as follows: in February 2011, beech seeds were subject to cold stratification in sand to fulfill dormancy so as to germinate promptly. After 12 weeks of stratification the germinated seeds were planted in 1.8 l pots. In every pot, two glass tubes were put at its sides in order to create a space for inoculum. Plants were grown for 6 months in ambient and enhanced CO_2 before inoculation with *Phytophthora* species in October 2011. Two variants of the experiment were carried out: the treatment inoculated with *Phytophthora* species and the control. Into all the pots with plants, 36 ml (2% of soil volume) of semiliquid medium was added, either infected with *Phytophthora* species (*Phytoptora* treatment) or sterile (control treatment). Then, all the pots with plants were partially submerged in water for 72 hours to stimulate *Phytophthora* sporangia production. The harvest of plants and confirmation of the presence of *Phytophthora* in soil and tissues took place six months after inoculation.

Comparison of seedlings growth and root morphological characteristics

The length [cm] of the main shoot and the main root of each plant were measured. For the comparison of root systems, two indicators for root morphological characteristics were selected: fine root length/mother root length (FRL/MRL) and fine root surface area (FRSA). Statistically significant differences between mean values of the length of above- and below-ground plant parts of beech seedlings and morphological characteristics of roots were examined. There were analysed FRL/MRL and FRSA indices based on data generated through scanning beech root systems with Epson 700 water scanner and using WinRhizo® software. The obtained data were subject to statistical analyses by means of the Kruskal Wallis nonparametric ANOVA test, at a significance level α=0.05, (Statistica v.10 by StatSoft). Since the data obtained did not meet requirements of parametric tests (homogeneity of variance, the same number of observation in groups and compliance with the normal distribution) the nonparametric tests were applied.

Re-isolation of *Phytophthora* species from soil with baiting (plant traps)

In order to confirm the presence of pathogens in the soil examined, it was attempted to re-isolate tested fungi one month and six months after inoculation. Earlier pathogen-inoculated soil was taken as an aggregate sample from the plant pots and put into plastic containers. Subsequently, the soil was covered with distilled water, in such a way that water surface was about 2 cm above the soil, and then the containers were left alone for 2 hours to allow debris to fall down to the bottom. Floating debris were removed with paper towel. Next, cleaned and dried leaves of 'Nova Zembla' rhododendron were placed on the surface of water in the containers as a baits for *Phytophthora* spores. After 5–7 days, there were observed the earliest spots on the surface of rhododendron leaves, and these were the sign of progressive infection with *Phytophthora* pathogens present in the soil. Infected leaves were collected, thoroughly cleaned with distilled water and blot dried on paper towels. Small fragments from the border between healthy and infected tissues were placed on the selective medium (PARP (Jung 1996) and incubated in 22°C for 1 week.

Re-isolation of *Phytophthora* from plant tissues

There was checked whether the pathogens were present in infected plant tissues. For this purpose, 5 beech seedling infected with investigated *Phytophthora* species and indicating the symptoms of dieback (yellow leaves), growing either at ambient or enhanced CO_2 concentrations were selected. The seedlings were thoroughly rinsed with water in order to remove soil from their surface and then roots, root necks and stem samples were taken from diseased seedlings. Five samples (app.2 mm long sections) of rotten root parts were taken and since disease symptoms not visible on other seedling parts, only one sample each was collected from stems and root necks. Tissue samples were surface-sterilized with 1%

sodium hypochlorite. Afterwards, small fragments (approximately 3×3 mm) were cut out with a scalpel and placed on PDA and on PARP selective media. Additionally, tissue fragments extracted from uninfected plants were placed on medium. Petri dishes were incubated at 22°C for 1 week. Mycelium growth was monitored every day.

Isolation of DNA from the plant tissues

Isolation of DNA from plant tissues (fine roots and root neck) was conducted with the use of GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich), according to the producer's instructions. In order to test DNA quality and quantity, electrophoretic separation of genomic DNA was carried out. Gel electrophoresis (1% agarose in 1xTBE buffer) was conducted for an hour at 100V. DNA was visualized using ethidium bromide under ultraviolet light and the results were saved with the use of GelDoc® system.

In order to detect DNA of *P. cactorum* and *P. plurivora*, a nested PCR technique was applied. In the first round, amplification with general primers for Oomycetes was carried out using primer pair DC6/ITS4 (White et al. 1990). The 25 µl PCR mixture (Taq PCR Master Mix, Qiagen) contained 1x Q buffer, 1x PCR buffer, 1.5 mM $MgCl_2$ (Qiagen), 0.2 mM of each dNTP, 0.15 µM of each primer (Oligo), 1 U of *Taq* polymerase (Invitrogen), 1 µl of genomic DNA, MilliQ water to the volume of 25 µl. PCR amplification conditions were set as follows: an initial denaturation temperature of 95°C for 3 min, followed by 25 cycles of denaturation temperature 95°C, primer annealing at 54°C for 30 s, primer extension at 72°C for 50 s and a final extension step at 72°C for 10 min.

One µl of above mixture was taken as a DNA template in the second round of nested PCR with primers specific for *P. cactorum* (ADR1 and ADF1 Lacourt et.al. 1997) and *P. plurivora* (CITR1 and CITR2 Nechwatal et. al. 2001). The composition of the PCR mixtures was as described above. PCR amplification conditions were set as follows: an initial denaturation temperature of 95°C for 3 min, followed by 35 cycles of denaturation temperature 95°C, primer annealing at 54°C for 30 s, primer extension at 72°C for 50 s and a final extension step at 72°C for 10 min. The PCR products were electrophoretically separated and visualised according to the above described conditions.

Results

The growth of beech seedlings under ambient and elevated CO_2 concentrations

The difference in growth of beech seedlings growing at different CO_2 concentrations were found, however – not statistically significant. The probability values obtained for shoot length (p-value 0.8819),fine root length (FRL) (p-value 0.087), FRL/MRL (p-value 0.132) and FRSA (p-value 0.082) did not prove statistically significant differences. The real average values of shoot length were: 9.43 cm (400 ppm CO_2) and 8.71 cm (800 ppm CO_2), those of of fine root length were 141.84 cm (400 ppm) and 199.66 cm (800 ppm), whereas FRL/MRL index values were 8.55 (400 ppm) and 10.67 (800 ppm), and FRSA – 27.57 (400 ppm) and 31.05 (800 ppm).

Damage caused by *Phytophthora* to beech roots

At ambient concentration of CO_2, there were no significant differences found between seedlings inoculated with *Phytophthora* and control plants. The p-values obtained were as follows: FRL – 0.611, with the mean value for the control – 141.84 and that for *Phytophtora* treatment – 150.89, FRSA – 0.469 with the mean for the control – 27.57 and for *Phytophtora* treatment – 28.89. Statistically significant difference (p-value is 0.035) was observed in case of FRL/MRL index with the mean value for the control – 8.55, and for *Phytophtora* treatment – 15.96 (fig. 1).

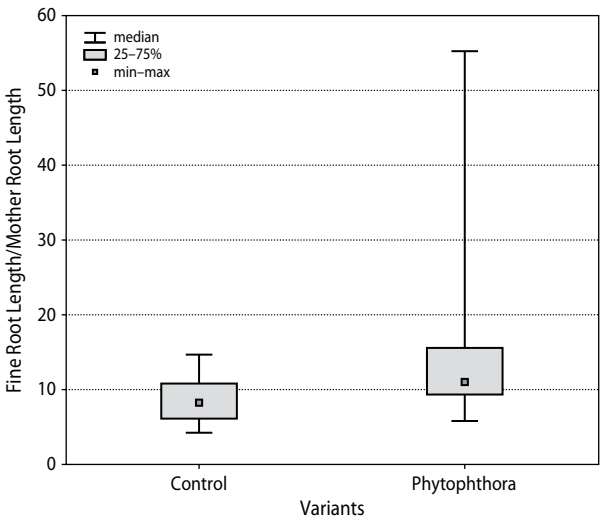

Figure 1. Comparisons of FRL/MRL index at ambient CO_2 concentration

In enhanced concentration of CO_2, statistically significant differences were observed in case of FRL (p-value = 0.039). The mean value for the control was 199.66, and for *Phytophtora* treatment – 153.34 (fig. 2). FRL/MRL p-value was 0.05, and the mean value for the control was 10.67 whereas for *Phytophtora* treatment it was 15.33 (fig. 3). In the treatment variant, the lowest values of each parameter were observed for seedlings inoculated with *Phytophthora* species, which showed fine root damage. In case of inoculated plant, fine root surface areas were also smaller when compared with control variant and this difference was insignificant (p-value = 0.059; mean value for the control – 31.05, and for *Phytophtora* treatment – 27.75).

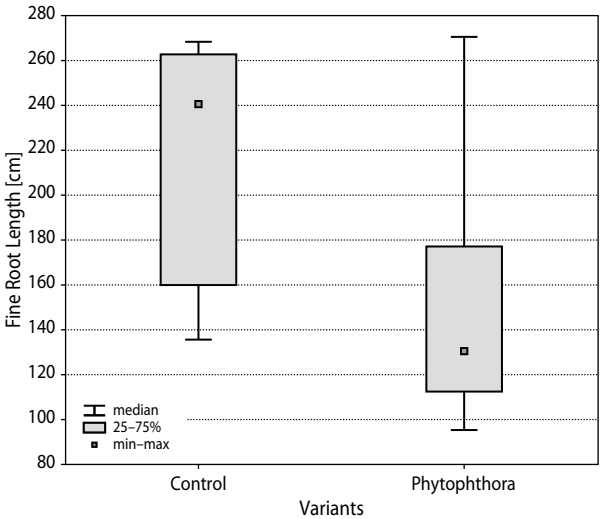

Figure 2. Comparisons of fine root length (FRL) at enhanced CO_2 concentration

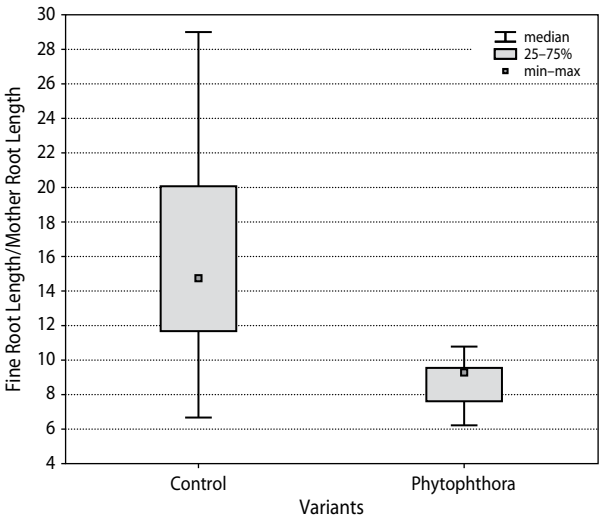

Figure 3. Comparisons of index of fine root length per mother root length (FRL/MRL) at enhanced CO_2 concentration

Re-isolation of *Phytophthora* species from the plant tissues and DNA analyses

There was performed re-isolation of *Phytophthora* from the roots and root necks of beech seedlings grown at enhanced CO_2 concentration and 60% of all the analysed samples showed the presence of this pathogen.. Both *Phytophthora* species (*P. cactorum* and *P. plurivora*) were isolated from a comparable number of beech seedlings (40% for *P. cactorum*, 60% for *P. plurivora*). At the same time, under ambient CO_2 conditions, pathogen re-isolation from plant tissues failed. Nevertheless, DNA analyses of the plant tissues examined, detected *P. plurivora* DNA in all the analyzed beech seedlings growing at enhanced CO_2 six months post inoculation and in 70% of analysed beech seedlings growing under ambient CO_2 conditions. .Identification of *P. cactorum* DNA in beech seedlings observed showed 30% and 70% of positive results in enhanced and ambient CO_2 concentrations, respectively. *Phytophthora* presence was not detected in the control treatment.

Discussion

Climate change could have impact on different plant diseases. Regardless of the effects of weather on plant diseases, a comprehensive analysis on how climate change will influence plant diseases that impact primary production in agricultural and forest systems has been up to date unavailable (Chakraborty et al. 2000). In the present study, after six months of the experiment, there were found no statistically significant differences between the growth of beech seedlings under the conditions of enhanced and ambient CO_2 concentration. Elevated CO_2 (800 ppm) had no effect on beech growth (length of above-ground parts)..This is not surprising, when beech growth pattern (usually-single flush in the spring) is taken into consideration. In the literature, there is a very little information on how a host plant (in this case beech) changes its behaviour under different environmental conditions. Henn

et al. (2000) and Stiling et al. (2003) found reduced herbivore feeding on beech and oak leaves of plants grown under elevated CO_2, while Percy et al. (2002) reported increasing herbivore damage, but no change as for rust infection by *Melampsora medusae* on aspen leaves under elevated CO_2. In the present, higher concentration of CO_2 increased damage caused by *Phytophthora* pathogens.

The development of *Phytophthora* in the fine roots and root collars of beeches growing under elevated CO_2 conditions of was investigated in this study. By comparing FRL/MRL) indexes, information about the numbers of fine roots could be elucidated. The lowest index values were obtained for beech roots infected with *Phytophthora* isolates, while the highest values (which indicated the large number of fine roots) were found in control trees. Despite a relatively short period of time between soil inoculation and manifestation of the symptoms of seedling damage, in the part of the roots (expressed in the length of fine roorts, the surface of fine roots and the mentioned above index of fine roots length per mother roots length). The inoculated seedlings which growth in the elevated carbon dioxide concentration had more significant damage of root systems. This phenomenon may result from the higher number of beech fine roots which developed under higher CO_2 concentration when compared with beech seedlings grown under ambient CO_2 conditions.

Growth patterns of specific colonies on the selective medium, as well as microscopic observations of characteristic hyphae confirmed the presence of *Phytophthora* phytopathogens in the tissues of infected beech trees. Generally, re-isolation of *Phytophthora* species from plant roots is very difficult, because the pathogens quickly damage the fine roots, which subsequently come off the mother roots. Then, the pathogens remain in soil (as endospores) and wait for another favourable period for their growth, e.g. periodical flooding (Erwin, Ribeiro 1996). In this study, under the conditions of elevated CO_2, beech seedlings produced more fine roots, which in turn were damaged by *Phytophthora* species, which considerably increased the chances of pathogen re-isolation from the plant tissues examined.

The above results are similar to those obtained by Fleischmann (2010), who also found altered root-shoot ratios and enhanced specific root tip densities. Author demonstrated that susceptibility of beech towards *P. citricola* was the highest when trees were grown under elevated CO_2. In subject literature, there has been no general agreement on how host-pathogen interactions change under different environmental conditions. The inoculation of seedlings and estimate the effect of elevated CO_2 on a *Phytophthora* disease was performed with tomato and *P. parasitica* by Jaw and Walling (2001). The results obtained by these authors indicated only a trend of increasing tolerance of tomato under elevated CO_2. Similarly, with the growth-differentiation balance hypothesis (Herms and Mattson 1992), elevation of atmospheric CO_2 in this study enhanced susceptibility of beech seedlings towards root pathogens *P. plurivora* and *P. cactorum*.

Conclusions

- There were found differences in growth of beech seedlings infected by *Phytophthora* species when growing in the conditions of altered CO_2 concentrations.
- Elevated CO_2 concentration*s* stimulate activity o*f Phytophthora* s*pecies (P. cactorum* and *P. plurivora*) which cause larger damage to the fine roots of beech seedlings.
- *Phytophthora* species were re-isolated from the roots and root collars of beech seedlings inoculated *via* soil. Pathogen presence was also confirmed by DNA analysis.

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