METAL UPTAKE BY SCOTS PINE (*PINUS SYLVESTRIS* L.) INFECTED BY *HETEROBASIDION ANNOSUM*

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Abstract. *Heterobasidion annosum (Fr.) Bref., also known as annosum root rot, is considered to be the most economically important forest pathogen in the Northern Hemisphere. This pathogen is the biggest threat to coniferous trees. This study was carried out in Lithuania (Kaunas city), in the experimental forest where the former agricultural field was afforested by Scots pine (Pinus sylvestris L.) stands 50 years ago. This engendered the spread of disease. Research was based on analysis of concentration of metals (Zn, Cu, Mg, K, Cd and Pb) in wood as well as in soil samples. Results showed significant higher concentrations of K and Mg in infected wood samples because of the decay caused by pathogen which had created a sink region in the infected tissues and that elements moved from the unaffected areas to the zone of infection.*

Keywords: *H. annosum, trace metals, macroelements, Pinus sylvestris L., transfer factor*

INTRODUCTION

Heterobasidion annosum (H. annosum) is one of the most common fungal diseases of conifers in Europe and the North America. Every year economic losses due to damage by *H. annosum* in Europe reach up to 790 million euro (Samil 2008). In Lithuania pine forest areas damaged by *H. annosum* are significantly larger than 100.000 hectares (Vasiliauskas 2001) and every year economic losses reach millions of litai.

Scots pines (*Pinus sylvestris* L.) are able to grow rapidly and produce timber of a high quality even in most barren sandy soils, where other trees would not be able to survive. In postwar period pine trees were afforested in mature pine glade, fire-damaged places, squares, shifting sands and soils formerly used for agriculture. This is precisely why fungal disease can spread easily. One of the reasons is that this type of soil does not have features appropriate for forest soils with their natural microfauna and microflora, which creates unfavorable conditions for spread of fungal disease (Vasiliauskas et al. 1966).

The life cycle of *H. annosum* is well known; it spreads via root grafts from infected trees or stumps to uninfected trees, causing root rot and/or butt rot, depending on the host species (Tainter

et al. 1996; Varese et al. 2003; Deacon 2006). The primary infection is mediated by basidiospores (Asiegbu et al. 2005). After *H. annosum* has colonized a stand of a tree, infection of neighboring trees occurs almost exclusively in the root region, either by spores which are washed into the upper horizon of the soil by rain and germinate on the roots, or by root contacts with a diseased tree (Schwarze 2000). *H. annosum* is a species complex consisting of several closely related species and intersterility (IS) groups (Olson 2006). Identification of infection by *H. annosum* in these centers is observed by several signs and symptoms (Schmitt 1989). Main symptom of *H. annosum* is fruit bodies (Fig. 2). The spores of *H. annosum* are released from the fruit bodies throughout the year and are carried by wind to colonize freshly cut stumps thus clear cutting and thinning of forest provide ideal conditions for its spread (Matthews 1991). Precluding infection of *H. annosum* silvicultural, biological and chemical control methods are applied in forest management. For silvicultural method – optimal spacing of tree planting is applied (Vasiliauskas 1975; Asiegbu 2005). There are several methods of chemical treatment against *H. annosum* as *Borax* (sodium borate), that acts as fungicide by preventing the formation of fungal spores (Housecroft 2007); *Propiconazole* – systematic foliar fungicide, which is translocated acropetally in the xylem (Roberts 1998); urea, which applied to a fresh stump surface and raises the pH > 7 in the adjacent wood tissues and prevents both the growth of *H. annosum* and the germination of basidiospores on wood (Johansson 2002). The most used biological control method for *H. annosum* is *Phlebiopsis gigantea* (*P. gigantea*), and it was one of the first commercially available agents for biological control of a plant pathogen (Van Driesche 1996).

When a tree becomes infected by a fungus, its natural defenses are triggered. The infection causes increased production of fungus inhibiting phenolic compounds and flavonoids, both at the site of infection and in other parts of the plant. The production and transport of these compounds is controlled in large part by the nutrition of the plant. Therefore, shortages of key nutrients such as K, Mn, Cu, Zn, and B reduce the amount of the plants natural antifungal compounds at the site of infection (Spectrum Analytic Inc. 2008). Some of the elements are known to be used as main compounds in fungicides (especially Zn and Cu). Macroelements K and Mg are essential and necessary in most of the biological processes of tree growth. Though, little is known about the distribution of elements in the infected trees and their interaction with decay. Some researches have been done in purpose to see the interaction between pathogen and macroelements as well as to see how applied microelements reduced diameter of mycelium colony. These researches were carried by Vasiliauskas (1964) in laboratory conditions. Results showed that Zn and Cu solutions have reduced growth of mycelium (Vasiliauskas 1964). Some authors (Muhamed et al. 1984) had investigated wood decay to see accumulation of macroelements as well as needles of infected pine tree (Tuner 1977).

The aim of the study was to observe the ability of infected tree to uptake trace metals and macroelements. Mg, K, Cu and Zn were chosen for analysis in re-

lation to their significance on the pine growth. Pb and Cd were observed due to lack of information about their behavior in infected pine wood.

1. MATERIAL AND METHODS

1.1. Study area

The study area is located in Lithuania, near the second largest city of Lithuania, in the experimental forest site at 54°53`12`` N, 24°04`33`` S (Fig. 1). The experimental site formerly was used for agriculture. After the land was no more sufficient for husbandry, the territory was taken under the project of afforestation in year 1959–1963 (Dubravos eksperimentinė mokomoji miškų urėdija 2009). Forest consisted of coniferous, mainly Scots pines along with deciduous impurities: Birch (*Betula* L.), Norway maple (*Acer platanoides*); other common vegetation such as: common wood sorrel (*Oxalis acetosella*), rowan (*Sorbus aucuparia*), woodland strawberry (*Fragaria vesca*), shield fern (*Dryopteris*), dandelion (*Taraxacum*), nettle (*Urtica*), red raspberry (*Rubus idaeus*). Soil type in the investigation site was sand and sandy loam.

1.2. Wood sampling

For analysis 10 infected pine trees and 10 pine trees without indicators of infection of similar age were chosen for analysis. The infected pines were identified according to inherent signs like fruit bodies (Fig. 2) and physical appearance. Trees were sampled using increment borer with diameter of 12 mm at pine stem high of 1.5 m. Coordinates of each tree were recorded and are presented in Figure 1.

1.3. Soil sampling and chemical analysis

Soil samples were collected in May 2009 near each sampled tree in prevailing wind direction. Two depths of soil sampling were chosen: top soil (0–20 cm) and subsoil (20–40 cm). Soil samples were placed in plastic bags and marked. For further analysis soil was dried and sieved (using 1 mm sieve). 20 g of each soil sample was weighted with electronic scales (± 0.2 g), placed in special bottles and filled with 100 ml of deionised water. Bottles were marked, tightly closed and placed into the shaker for one hour to agitate. After shaking samples were left to settle for 30 min before filtration. pH of soil solution was measured using WTW (pH 538) pH meter. For reliability each of the samples was measured 3 times along with five blank samples.

For measuring total organic carbon (TOC) in soil 0.4 g of each soil sample was weighted. For analysis TOC analyzer from Shimadzu (TOC-VCSN) was used. The total carbon content was obtained using Equation 1 (ICP Forest manual 2006):

$$w_{Ct} = 1000 \times \frac{m_2}{m_1} \times 0.2727 \quad (1)$$

Where *wct* – total carbon content (g kg^{-1}) on basis of air dried soil; m_1 – mass (g) of test portion (0.4 g); m_2 – mass (g) of released CO_2; 0.2727 – conversation factor for CO_2 to C (ICP Forest manual 2006).

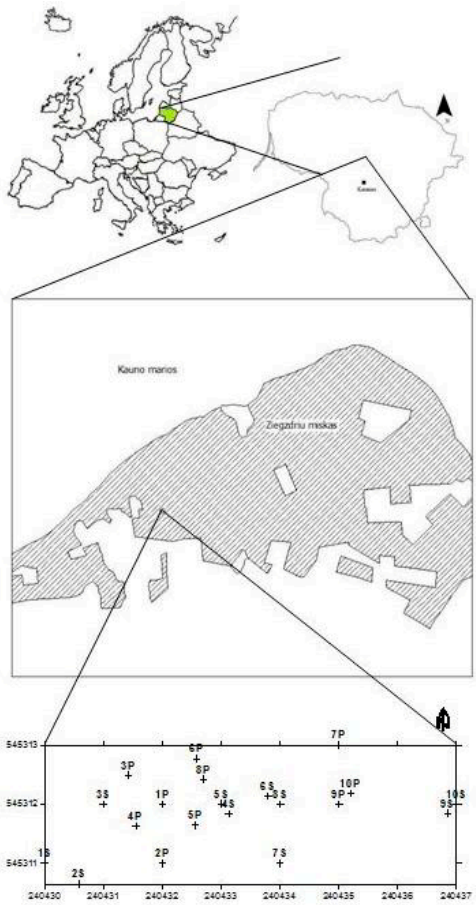

Fig. 1 Location of the sampling territory in the map (Scale 1:50000). Distribution of sampled Scots pine stands along the territory (P – infected trees; S – control trees)

Fig. 2 fruit body of *H.annosum* on stems of one of the sampled Scots pine (Kaunas 2009)

Bioavailable fraction of Zn, Cu, Mg, K, Cd and Pb in soil was analyzed to determine the pool of available metals in the soil. Bioavailable fraction of metals was analyzed in the soil solution prepared for pH analysis. Analysis was performed using graphite furnace (BUCK SCIENTIFIC – 220 GF).

0.5 g of soil was mixed with hydrochloric acid (37 %) and nitric acid (65 %) in ratio 1:3. Mixture was di-gested in the microwave digester Milestone ETHOS. After that solution was filled with deionised water to the mark of 50 ml. For the analysis flame atomic absorption spectrometer (FAAS) (BUCK SCIENTIFIC – 220 GF) was used.

Pinewood cores were incinerated to ashes at 450°C in ceramic plates. Total metal concentrations in wood samples were measured mixing ashes with hydrochloric acid (37 %) and nitric acid (65 %) in ratio 1:3 and after digested in the microwave digester Milestone ETHOS. Total concentration was measured using flame atomic absorption spectrometer (FAAS) (BUCK SCIENTIFIC – 220 GF).

Transfer factor (Tf) and percentage of extractable metals

Tf is a convenient method of expressing the relative ease with which elements in soils (total concentrations) are taken up by plants and accumulated in their above – ground tissues (Selinus et al. 2005). The coefficient is obtained using Equation 2:

$$Tf = \frac{C_{wood}}{C_{soil}} \quad (2)$$

where *Tf* – transfer factor; *Cwood* – total concentration of metal in wood sample (mg kg^{-1}); *Csoil* – total concentra-tion of metal in soil sample (mg kg^{-1}, DW).

Percentage of extractable metals was calculated using Equation 3:

$$Ext(\%) = (C_{total} / C_{bioavailable}) \cdot 100\% \quad (3)$$

where *Ext(%)* – percentage of extractable metals; *Ctotal* – total metal concentration in soil (mg kg^{-1}, DW); *Cbioavailable* – metal concentration in soil solution (mg kg^{-1}).

Statistical analysis

Experimental data were processed using Microsoft Excel 2003. Mean values were estimated, as well as standard error (SE). Significant difference and analysis of variance (ANOVA – one way) were carried out using the statistical programs StatistiXL and STATISTICA 7 software.

2. RESULTS

2.1. Soil properties. pH and TOC

pH of soil varied from 4.93 to 6.48 along the whole territory of investigation. There was not observed any significant difference between values of pH near infected and control sites. Though the difference between pH values in different soil layers were estimated: pH was lower in top soil (0–20 cm) than in subsoil (20–40 cm). The mean pH value – 5.34; soil type is classified as subacid and is sufficient for coniferous growth; though it creates a high condition for spread of *H. annosum*. Soil pH affects soil properties, nutrient availability and plant nutrition.

The values of total organic carbon (TOC) ranged from 0.392 to 9.857 g/kg. No significant difference was observed between values near infected or control trees, though there was a difference between TOC of different soil layers: TOC was from 50 to 70 % higher in subsoil (20–40 cm) than in top soil (0–20 cm).

2.2. Total metals in soil

As shown in Table 1 there are significant differences between control and infected samples comparing total metal concentration in soil. Concentrations of Mg in infected soil were from 427 to 599 mg kg^{-1} (average of two depths) and in control – from 397 to 620 mg kg^{-1}; for K these concentrations were from 317 to 1257 mg kg^{-1} and from 644 to 1938 mg kg^{-1} respectively. Comparing average values Mg did not showed significant difference between infected and control sites, while K concentrations were significant higher in control soil samples. Concentrations in control soil samples were 1.5 times higher comparing with infected.

Concentrations of trace metals were following; Zn – from 18.8 to 48.3 mg kg^{-1} in infected soil and from 26.2 to 95.5 mg kg^{-1} in control, for Cu these concentrations were 0.018 – 0.028 and 0.008 – 0.025 mg kg^{-1} respectively. Difference between infected and control soil samples of Zn

were not significant different, though were a bit higher in control soil samples. Concentrations of Cu showed significant difference and were higher in infected samples 1.5 times. Concentrations of Pb did not showed significant difference between samples and were relatively low: 1.9 – 6.32 mg kg^{-1} in infected and 2.1 – 4.1 mg kg^{-1} in control, but concentrations of Cu were unexplainable high in control soil samples: from 0.02 to 0.53 mg kg^{-1}.

Estimated total concentrations of metals in soil (except macroelements) were compared with background values of soil (HN 60:2004). Concentrations of Zn and Cd were exceeded. Though concentration of Cd in soil samples near control trees were inexplicably too high and even 7 times bigger than in soil samples near infected trees. This error could appear due to inaccuracy during investigation or possible single pollution case in the point of sampling.

Table 1. Total metal concentration in soil samples (n=10). Values are mean (mg kg^{-1} DW) ± S.E. Means and S.E. of n=10 trees and results of one-way ANOVA for each metal. Asterisk shows significant difference at p<0.05 and p<0.01. Values compared with soil background concentrations

	Soil	Background
Zn		
Infected	31.19 ± 0.09	
Control	40.48 ± 0.25	26.00
F	1.22	
Cu		
Infected	0.024 ± 0.0004	
Control	0.016 ± 0.001	8.1
F	14.83*	
Mg		
Infected	499.86 ± 0.63	
Control	492.82 ± 0.74	-
F	0.05	
K		
Infected	826.47 ± 2.63	
Control	1282.13 ± 3.82	-
F	9.66**	
Pb		
Infected	3.37 ± 0.01	
Control	2.99 ± 0.01	15.00
F	0.56	
Cd		
Infected	0.038 ± 0.002	
Control	0.25 ± 0.0016	0.15
F	18.30**	

2.3. Bioavailable metals in soil

Bioavailable metal concentrations (Table 2) for macroelement K were significantly higher in infected (from 7.13 to 23.74 ml/l) than in control (from 4.46 to 22.54 ml/l). Concentrations of Mg did not show significant difference and bioavailable forms varied similary along the site of investigation (from 1.05 to 6.86 ml/l).

Concentrations of bioavailable Cu were relatively low but had significant difference comparing infected and control soil samples (Table 2). Metals Zn and Pb didn't show significance comparing infected and soil, while Cd was significantly higher in infected soil samples and varied from 0.0 to 0.004 ml/l.

Table 2. Bioavailable metal concentration in soil samples (n=10) and Ext(%). Values are mean (mg kg^{-1} DW) ± S.E. Means and standard errors of n=10 trees and results of one-way ANOVA for each metal. Asterisks shows significant difference at p<0.05, p<0.01 and p<0.001

	Soil	E_{xt}(%)
Zn		
Infected	0.46 ± 0.004	1.72 ± 0.68
Control	0.22 ± 0.001	0.67 ± 0.12
F	3.56	4.72*
Cu		
Infected	0.001 ± 0.00001	4.65 ± 1.66
Control	0.0002 ± 0.000002	2.12 ± 0.86
F	5.82*	3.68
Mg		
Infected	2.67 ± 0.01	0.55 ± 0.08
Control	2.41 ± 0.02	0.46 ± 0.09
F	0.16	1.1
K		
Infected	13.42 ± 0.06	1.83 ± 0.41
Control	7.82 ± 0.05	0.69 ± 0.15
F	5.10*	13.78***
Pb		
Infected	0.022 ± 0.0003	0.62 ± 0.17
Control	0.008 ± 0.0001	0.25 ± 0.06
F	2.96	7.64**
Cd		
Infected	0.0009 ± 0.00001	1.74 ± 0.78
Control	0.00004 ± 0.000001	0.03 ± 0.02
F	4.42*	9.62**

2.4. Total metals in wood

In all cases metals concentrations in wood samples were higher in infected trees than in not infected (Table 3). For macroelements these differences were most noticeable in concentration of K: from 6.5 to 207.5 mg kg^{-1} in infected and from 22.9 to 55.6 mg kg^{-1} in control samples of wood. The average concentration of K in infected pine wood was 3 times higher than in control. Mg also showed significant higher concentrations in infected pine wood than in control: from 3.8 – 94.8 mg kg^{-1} and from 11.3 to 55.6 mg kg^{-1} respectively.

Trace metal Zn showed 1.7 times higher concentrations in infected pine wood than in control and varied from 0.1 to 2.81 mg kg^{-1} in infected and from 0.47 to 1.64 mg kg^{-1} in control samples. Concentration of Cu did not show differences and varied from 0.01 to 0.49 mg kg^{-1} in infected and from 0.07 to 0.65 mg kg^{-1} in control samples of wood. Concentrations of Pb and Cd were low, though Cd concentrations were significant different comparing infected and control wood samples.

Table 3. Total metal concentration in wood samples (n=10). Values are mean (mg kg^{-1} DW) ± S.E. Means and S.E. of n=10 trees and results of one-way ANOVA for each metal. Asterisk shows significant difference at p<0.05, p<0.01 and p<0.001

	Wood
Zn	
Infected	1.59 ± 0.01
Control	0.93 ± 0.03
F	5.98*
Cu	
Infected	0.21 ± 0.001
Control	0.17 ± 0.002
F	0.32
Mg	
Infected	42.94 ± 0.25
Control	20.57 ± 0.13
F	6.27**
K	
Infected	95.83 ± 0.50
Control	32.19 ± 0.10
F	15.32***
Pb	
Infected	0.027 ± 0.0001
Control	0.016 ± 0.0001
F	3.38

	Wood
Cd	
Infected	0.007 ± 0.00004
Control	0.004 ± 0.00002
F	5.24*

2.5. Transfer factor

The highest Tf was observed in Cu and varied from 0.56 to 34.69 in infected sites and from 2.67 to 29.24 in control sites (Table 4). Based on literature it could be classified as element that is accumulated intensively, though data can be interpreted differently. The concentration of Cu was found higher in wood samples than in soil. This could be observed because of possible other sources of Cu (e. g. atmospheric deposition). Other metals showed similar abilities for bioaccumulation, which could be classified as elements with medium accumulation. All elements, except Cu, appeared to have higher Tf from soil to infected trees than to control. Significant difference was observed in concentration of Mg: from 0.008 to 0.255 for infected sites and from 0.02 to 0.12 for control.

Table 4. Transfer factors (n=10). Values are mean (mg kg^{-1} DW) ± S.E. Means and standard errors of n=10 trees and results of one-way ANOVA for each metal. Asterisks shows significant difference at p<0.05

	Tf
Zn	
Infected	0.05 ± 0.0003
Control	0.03 ± 0.0002
F	2.42
Cu	
Infected	9.39 ± 0.07
Control	11.16 ± 0.12
F	0.45
Mg	
Infected	0.09 ± 0.0005
Control	0.04 ± 0.0002
F	4.54*
K	
Infected	0.14 ± 0.0008
Control	0.03 ± 0.0002
F	2.00
Pb	
Infected	0.01 ± 0.00005
Control	0.006 ± 0.00004
F	2.10
Cd	
Infected	0.25 ± 0.0025
Control	0.07 ± 0.0012
F	1.03

3. DISCUSSION

According to Fedorov (Федоров 1984), physiological activity of roots affected by *H. annosum* is less developed, and the amount of alive roots is 6 times less than in healthy tree. Because of that roots can become less capable to uptake water and elements so tree might wither. Renneerfelt and Tamm (1962) investigated the content of nutrient in needles of tree infected by *H. annosum* but found no difference in the nutrient level (Федоров 1984; Tuner 1977). This also could be due to that concentrations of metals are translocated mostly in needles (Rothpfeffer et al. 2007) and higher concentrations may not reflect the exact differences.

Our investigation has shown that infected Scots pine stands had bigger concentrations of metals than control, but not in all cases difference was significant. The major difference was observed for macroelements Mg and K The reason for this difference could be that the tree colonized by wood rotting fungi, creates

a sink region in the infected tissues and that elements move from the unaffected areas to the sink (Muhamad et al. 1984). Taking in to account this hypothesis we can see that most of the analyzed wood samples were really marked with observable decay (Fig. 3 a, b).

Fig. 3. a) sample from the pine infected by *H. Annosum*; b) – sample from control pine

Not all of the collected samples had significant signs of decay; others had discoloration of wood that is also important, because this part of wood also shows higher abilities of accumulating elements than clear wood, though less than decayed wood (Muhamad et al. 1984).

The most substantial significance differences between infected and control trees concentrations was in K diverge. The higher K concentration in wood could indicate a physiological response of the tree to the disease. According to Rykowski (1981) higher K concentration attributes to defensive mechanism of the tree due to the role of K in the activation of enzymatic system. Though Renneerfelt and Tamm (1962) associate high level of K in infected tree tissues because of the fungi ability accumulate K. Basidiomycete fungi have been shown to accumulate large quantities of K within rhizomorphs (Mallett et al. 1998).

Other metals that showed significant differences among treatments were Mg, Zn and Cd. Higher concentration of Mg in infected trees could be related to the same reasons as K as it is also essential nutrient. Though concentrations of Mg in wood were lower that could be related to low concentration of Mg in soil (Adriano 2001). Root pathogen produce trivial hyphal growth into soil or with marked ectotrophic growth may, in contrast to their mycorrhizal relatives, use extanerous nutrients to the detriment of their hosts (Toussoun et al. 1990).

The uptake of metal was identified by using Tf showed significant difference for K and for Mg. This could also be explained relating the fungi ability to accumulate nutrients. Tf were also high for Zn and Cd. For Cu Tf there was not found any significance thought the Tf was really high. This could be because of possible other sources of Cu besides of soil.

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METALO SUGĖRIMAS ŠAKNINE PINTIMI UŽKRĖSTOSE PAPRASTOSE PUŠYSE

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Santrauka

Šakninė pintis (H. annosum) – ekonomine prasme viena svarbiausių miško ligų sukėlėjų Šiauriniame Pusrutulyje. Tai daugiausia žalos sukelianti liga spygliuočiams. Tyrimas buvo atliktas Lietuvoje, Kauno mieste, eksperimentiniuose Dubravos miškuose, kur pušynas buvo apželdintas ant buvusios dirbamos žemės prie 50 metų. Tai buvo viena iš priežasčių paskatinusių ligos plitimą. Tyrimų metu buvo analizuojami metalai (Zn, Cu, Mg, K, Cd ir Pb) medienos ir dirvožemio ėminiuose. Tyrimai parodė reikšmingus skirtumus tarp pažeistų ir kontrolinių medienos ėminių K ir Mg koncentracijose. Tai siejama su medžio puviniu, kuris sudaro sritį pažeistame audinyje ir elementai juda nuo nepažeistos vietos puvinio link.