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Occurrence and Diversity of Soilborne Phytophthoras in a Declining Black Walnut Stand in Hungary

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Abstract – The paper reports on the occurrence and impact of *Phytophthora* species in a declining eastern black walnut (*Juglans nigra*) stand in West Hungary. The health condition of the trees was investigated and soil samples were taken from the rhizosphere of the trees two times per year in 2011 and 2012 in order to isolate *Phytophthora* species. Altogether 20 trees were selected for investigations. The species identity of the isolates was determined by morphological and molecular methods. *Phytophthora cactorum* and *Phytophthora plurivora* were found as supposedly responsible for the decline of the trees. The abundance of the two species was changing at the different sampling times, presumably due to the different weather conditions. The intraspecific diversity of both species was estimated based on the ITS1-5.8S-ITS2 sequences of the isolates.

eastern black walnut / *Phytophthora cactorum* / *Phytophthora plurivora* / tree decline

Kivonat – Phytophthora fajok gyakorisága és diverzitása egy pusztuló feketedió állományban Magyarországon. A tanulmány egy pusztuló nyugat-magyarországi fekete dió (*Juglans nigra*) állományban előforduló *Phytophthora* fajokról, és azok faállományra gyakorolt hatásáról tudósít. A szerzők vizsgálták a faállomány egészségi állapotát, illetve talajmintákat gyűjtöttek a fák gyökérzónájából a *Phytophthora* fajok kitenyésztése céljából. A vizsgálatokat 20 megjelölt fán végezték, 2011-ben és 2012-ben, évente 2–2 alkalommal. Az izolátumok azonosítása morfológiai és molekuláris genetikai módszerekkel történt. *Phytophthora cactorum*-ot és *Phytophthora plurivora*-t találtak, mint a pusztulás valószínűsíthető okát. A két faj gyakorisága eltérő volt a különböző mintavételi időpontokban, feltehetően az eltérő időjárási viszonyok miatt. A két faj diverzitását az izolátumok ITS1 – 5.8S – ITS2 szekvenciái alapján becsülték.

fekete dió / *Phytophthora cactorum* / *Phytophthora plurivora* / fapusztulás

1 INTRODUCTION

Some species of the genus *Phytophthora* (*Oomycota*) are harmful and destructive pathogens of forest trees (Hansen 2008).These species cause root rot, bleeding cankers or wilting symptoms on susceptible forest trees (Erwin – Ribeiro 1996). There are soilborne, waterborne and aerial species within this genus. Inside a forest, or between woodlands, soilborne and waterborne species can spread via irrigation water, ground water or splashes. The spores of the aerial species are delivered by the wind. Phytophthoras can easily spread between

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countries or continents with infected plants or soil. Twenty-one percent of the invasive plant pathogens in Europe belong to the genus *Phytophthora* (Santini et al. 2012). The impact of these invasive pathogens is unpredictable (Brasier – Webber 2010). Under favourable conditions, the ecological and economical loss might be huge.

Up to 2010, 98 formally described species and 2 species hybrids belong to the genus *Phytophthora* (Érsek – Ribeiro 2010). The number of the described species grew and expectedly will continue growing because of multiple reasons: the available molecular tools enable a quick and accurate identification, the number of *Phytophthora* research groups and the attention on forest Phytophthoras grows worldwide, and also because of the recent evolutionary processes within the genus (Érsek – Ribeiro 2010).

Since the beginning of the 20th century, Phytophthoras have been responsible for many diseases and declines in European forests. The ink disease of sweet chestnut spread as an epidemic in the 1920–1940s and beyond the chestnut blight caused by *Cryphonectria parasitica*, it is still one of the main diseases of *Castanea sativa* (Vannini – Vettraino 2001). Since the 1990s, an upswing is present in the number of epidemics caused by *Phytophthora* species. *Phytophthora cinnamomi* endangers a uniqe agroforestal ecosystem, the so called 'dehesa' in the Iberian Peninsula by killing the holm oak (*Quercus ilex*) and cork oak (*Quercus suber*) since 1991 (Brasier 1996; Moralejo et al. 2009). The infected trees show wilting symptoms and die within a few years. The disease is spreading continuously.

A decline was noticed in the deciduous oak forests of North- and Central-Europe in the mid of the 1990s. The climatic instability, the acid soils with moderate water conditions and *Phytophthora* species, like *P. cambivora, P. citricola, P. gonapodyides, P. cactorum, P. syringae, P. europaea* and mainly *P. quercina* played a role in this complex decline (Jung et al. 1999, Jung et al. 2000, Balci - Halmschlager 2003).

In 1993, a decline of riverside alders *(Alnus glutinosa)* was noticed in Great Britain. The trees showed wilting symptoms. On the stem, cankers and tarry exudations were observed (Brasier et al. 1995). An unknown *Phytophthora* was isolated from the infected bark samples. The pathogen caused an epidemic across Europe. In 1995, it was found in Germany, Belgium, in the Netherlands and France, in 1996 also in Austria and Hungary (Cech 1997, Szabó et al. 2000). The molecular analysis of the isolates showed that it is a natural, interspecific hybrid. It was formally described as *Phytophthora alni* in 2004 (Brasier et al. 2004).

Many *Phytophthora* species were isolated from bleeding cankers found on Europaean beech *(Fagus sylvatica)* trunks or soil samples from the rhizosphere of declining beech trees since 2000. These species are *P. cactorum, P. cambivora, P. citricola, P. plurivora* and in Great Britain, 2 invasive quarantine species, *P kernoviae* and *P. ramorum* (Brasier et al. 2005, Weiland et al. 2010, Jung 2009). The last two species can became an extreme threat to the European and North-American forests. Pedunculate oak (*Quercus robur*) can be infected by *P. kernoviae* too (Brasier et al. 2005), while many other tree species, like North-American coastal live oaks, Douglas-fir (*Pseudotsuga menziesii*), coast redwood (*Sequoia sempervirens*) and yew (*Taxus baccata*), and ornamental shrubs like *Viburnum spp*., *Rhododendron spp*., *Syringa vulgaris*, *Pieris spp*. and *Vaccinium spp*. are also susceptible for *P. ramorum* (Grünwald et al. 2008). However, till 2009 the *P. ramorum* infections in European forests were local, mainly near highly susceptible viburnums and rhododendrons. An epidemic similar to the North-American Sudden Oak Death was noticed in 2009 in Japanese larch (*Larix kaempferi*) plantations in South-West England. This host jump elucidates the potential threats of invasive pathogens (Brasier – Webber 2010).

According to a previous research of the Institute of Sylviculture and Forest Protection, (University of West Hungary, Hungary) *Phytophthora* species infect common alder (*Alnus glutinosa*), eastern black walnut (*Juglans nigra*), sessile oak (*Quercus petraea*) and Turkey oak (*Quercus cerris*) stands in Hungary (Szabó – Lakatos 2008, Szabó et al. 2013).

The genus *Juglans* consists of ~21 taxa, with an extensive distribution from East Asia to the Americas. The black walnuts (Section *Rhysocaryon*) are native to the Americas. They include six North-American, three Central American and four South American species. An other American species (*Juglans cinerea*), native to eastern North America belongs to the section Trachycaryon. The English walnut (*Juglans regia*, Section *Juglans*) is the only native species in Europe. Its distribution is ranging from Europe to China and the Himalayas. The other species belonging to the Section *Cardiocaryon* are native to East Asia (Aradhya et al. 2007). English walnut is the most widely cultivated species from the genus throughout the world for nuts and also for timber production (Belisario – Galli 2013). However, black walnuts and hybrid rootstocks (i. e. Paradox rootstock) are widely cultivated, economically important species, too.

J. regia and *J. nigra* are two economically important hardwood species in Hungary. They grow on marshlands and riparian sites. Although the stands of eastern black walnut as an exotic tree species cover only 0,5% of Hungary's forest area, the timber of this species is valuable for furniture industry (Molnár – Bariska 2006).

The two closely related species share some common diseases. One of these is the *Phytophthora* root and collar rot of walnuts, which is an increasing cause of walnut loss in Europe and also in North America (Belisario – Galli 2013).

Twelve *Phytophthora* species are known as pathogens of walnut species, affecting seedlings and also mature trees worldwide. These species are *P. cactorum*, *P. citricola*, *P. cinnamomi*, *P. citrophthora*, *P. cryptogea*, *P. megasperma*, *P.cambivora*, *P. drechsleri*, *P. hedraiandra*, *P. nicotianae*, *P. palmivora* and *P. plurivora* (Mircetich – Matheron 1983, Belisario – Galli 2013).

There are no specific symptoms of *Phytophthora* infection in *Juglans* species. Laboratory methods are required to identify the cause of the decline. *Phytophthora* species can cause various disease symptoms on different parts of the tree. In the crown, wilting, small, yellowish leaves or sudden death can be observed. On the stem, bleeding cankers, tary spots or also large, dark, flame-shaped areas may be present. A main symptom of the disease is the rot of the root system. Usually the young feeder roots die first which is later followed by the death of the older roots (Belisario – Galli 2013). The development of visible symptoms depends always on the amount of infected roots, which is in context with the inoculum density of the soil. It can be a sudden death during summertime or a slow decline with a progressive reduction of foliage and fruit production over several years (Belisario – Galli 2013). Sudden death is mainly characteristic to infections caused by *P. cinnamomi* (Belisario – Galli 2013). In the case of a slow wilting, the infection may be undetected in the first years. Some trees also can survive the infection without notable crown symptoms (Belisario – Galli 2013).

The distribution of the pathogen, the infections and the disease severity is in context with the soil moisture and the duration of soil saturation (Belisario – Galli 2013). However, trees can be infected also by occasionally occurring floodings (Vettraino et al. 2003).

In the early 2000s, decline of eastern black walnut (*Juglans nigra*) stands was observed in Hungary, in South-Danubian floodplain forests in Gemenc. The canopy of over 80 years old trees showed wilting symptoms despite of adequate soil humidity and lack of any other visible reason of the decline. *Phytophthora* species were isolated from the soil. *P. cactorum* and *P. plurivora* were the most frequently occurring species. The pathogenicity of these species was confirmed by inoculation of black walnut seedlings (Szabó – Lakatos 2008). Since that time similar symptoms were observed in old black walnut stands in some other regions of Hungary, also in the north-western part of the country.

Many investigations have been done in the last decades in order to better understand the *Phytophthora* disease of English walnut. Matheron and Mircetich pointed out that the

susceptibility of *J. regia* seedlings against *P. citricola* shows seasonality. Their susceptibility is higher in summer and in the beginning of autumn than from late autumn to spring (Matheron – Mircetich 1985a). Vettraino et al. showed that the five most frequently isolated Phytophthoras cause different symptoms under the same conditions. According to their study, *P. cinnamomi* is the most pathogenic species against English walnut, *P. cactorum* and *P. cambivora* are slow colonizers which damages also lateral fine roots, while *P. citricola* causes the rot of lateral fine roots, but does not damage the taproot (Vettraino et al. 2003).

Our aims were to study the changes of health condition of an affected stand, the diversity of soilborne *Phytophthora* community in the rhizosphere of the trees, to test the pathogenicity of the isolated species against *J. nigra* seedlings and to study whether there is a seasonality or not in the *Phytopthora* composition of the soil of the infected trees.

2 MATERIALS AND METHODS

2.1 Sampling

Twenty black walnut trees were selected for sampling in a 73 years old marshland forest near Kapuvár, Hungary. The site investigated is on alluvial meadow soil with periodic water effect.

The health condition of the trees was evaluated based on the crown symptoms using the following 4-pointed scale:

1. Healthy crown.
2. Less than 20% of the crown is dying. Some leaves are yellowish.
3. 20–50% of the crown is dying. Leaves with yellowish discolouration in larger groups.
4. More than 50% of the crown is dead. Yellow leaves in large groups, or the remaining foliage is yellow.

Soil samples were taken from the rhizosphere of each investigated tree with a final volume of 1 litre. The sampling was done at four different point within 1 metre around each tree, from a depth of 5–30 centimetres. The aliquots were mixed.

2.2 Isolation

A quantity of approximately 250 mg from each mixed soil sample was flooded with 500 ml distilled water in a flat plastic container of a volume of 2 litres. The isolation of Phytophthoras was performed by using healthy, freshly picked, young *Rhododendron* and *Prunus laurocerasus* leaves as baits (Themann – Werres 1998, Nagy et al. 2000). The leaves were surface-disinfected with 10% NaOCl solution and rinsed in water before use. The samples were incubated in 22 °C, on daylight. Necrotic spots appeared on the leaves within 2-4 days. A piece of a size of 5x5 mm was cut from the infected leaf sections and put on the surface of *Phytophthora* selective agar plates (*Figure 1*). The medium used contained 1,5% malt extract and 2% bacteriological agar. Ampicillin (250 mg/l), Benomyl (15 mg/l) and hymexazol (50 mg/l) were added to the medium before use.

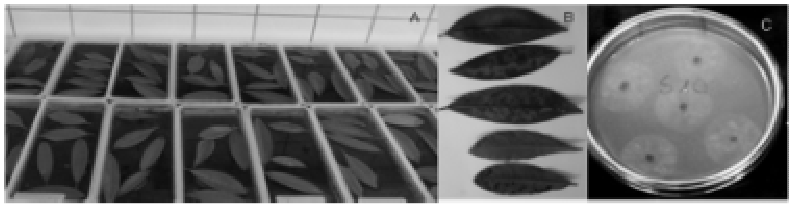

Figure 1. The method of isolation: A: leaf baits on the soil samples B: Characteristic lesions on the baits C: Phytophthora colonies on Phytophthora-selective agar media

2.3 Species identification

The species identification was done by using morphological features and ITS sequences.

The morphological features were studied on cultures grown on carrot agar (Brasier 1969), at 20°C, in the dark. The formation of sporangia was induced by non-sterile soil solution (Jeffers and Aldwinckle 1987). Daily growth rate, colony patterns and morphology of microscopic structures were investigated (Erwin – Ribeiro 1996).

The molecular identification of the isolates was made by amplifying and sequencing the ITS1-5.8S-ITS2 region of the rDNA of selected isolates. Pure mycelial cultures were used for DNA extraction or direct PCR. The DNA extraction and the PCR was made with the REDExtract N-Amp Plant Kit (Sigma – Aldrich), or with the PHIRE Plant Direct PCR Kit (Thermo Scientific) according to the user's guide of the manufacturers. ITS6 and ITS4 primer pair (Cooke – Duncan 1997) was used for PCR, in an Eppendorf Mastercycler Personal PCR machine. PCR conditions were as described by Cooke and Duncan (1997). The resulting PCR products were purified with EZ-10 Spin Column PCR Purification Kit (BioBasic Inc.), according to the user's guide of the manufacturer. The purified products were sequenced in both directions. Sequences were corrected and aligned with the ClustalX software. Homologs were searched in the GenBank (NCBI database) using BLASTN software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4 Estimation of the genetic diversity

The genetic diversity of the isolated species was estimated based on the ITS1 – 5.8S – ITS2 sequences. The sequences were compared by using the Clustal X software. Only complete, error-free sequences were used for the comparison.

2.5 Pathogenicity tests

Two strains were used for pathogenicity tests. A *P. plurivora* strain (202a) isolated in September 2011 and a *P. cactorum* strain (174/2) isolated in June 2011 from the studied black walnut stand. The colonies used were 14 days old. They were grown on Potato-Dextrose Agar plates (39g/L, Microtrade Ltd., Budapest, Hungary).

3,5 months old black walnut seedlings were infected. The seedlings were grown from disinfected seeds in containers. The soil used for planting was investigated with the above mentioned leaf baiting method and proved to be free of Phytophthoras. Eight seedlings/isolate and eight seedlings as control (totally 24 seedlings) were used for the tests.

The infection was done on 8th September 2012. A wound of a diameter of 5 mm was made on the base of the stem and a piece of a 5mmx5mm size from the pathogen colony was put into the wound. After that, the wound was closed with plastic paraffin film (Parafilm® Pechiney Plastic Packaging Company). The wounds on the control seedlings were closed with Parafilm without any infection.

The seedlings were watered as it was necessary. They were overwintered in a frostfree, closed chamber. The mean temperature and the humidity of the chamber fluctuated according to the weather conditions outside. Their parameters (length and diameter of the shoots, length and width of the roots, health condition of the shoots and roots and especially the length and width of the bark necrosis caused by the pathogens on the stem) were measured after 10 months, in July 2013.

2.6 Data analysis

The health condition datasets were analysed with the IBM SPSS 20 Software (IBM Corp. 2011). We used Kruskall-Wallis and Mann-Whitney tests to see the change in the health condition of our sampling site.

Data resulted from the pathogenicity tests were also analysed with the IBM SPSS 20 Software. The Gaussian distribution of data sets was tested with the Shapiro-Wilks method. The homogenicity of variances of the variables was tested with Levene statistic, based on the median. To test differences between the two infected and the non-infected groups, One-Way ANOVA was used. Independent Samples T-test was used to test differences between the two infected groups in case of necrosis area data sets.

In order to understand the changes in species composition, daily temperature and precipitation data of the region were collected from the database of the Hungarian Meteorological Service (http://www.met.hu/idojaras/aktualis_idojaras/napijelentes/). Walter-Lieth diagrams were constructed from these data with a free, online software (http://www.zivatar.hu/script.php?id=walter-lieth).

3. RESULTS AND DISCUSSION

3.1 Changes in the health condition

The health condition of the trees (*Figure 2*.) got significantly (*P = 0,023*) worse during the experiment according to the Kruskal-Wallis test. Healthy tree could be found only at the very first investigation (1 tree). The number of trees belonging to the 2nd category decreased from 9 to 3 trees, while the number of trees in the 3rd and especially in the 4th category increased (from 7 to 8 in case of 3rd, and from 3 to 9 in case of 4th category). However, we couldn't find any dead trees till the end of the monitoring period.

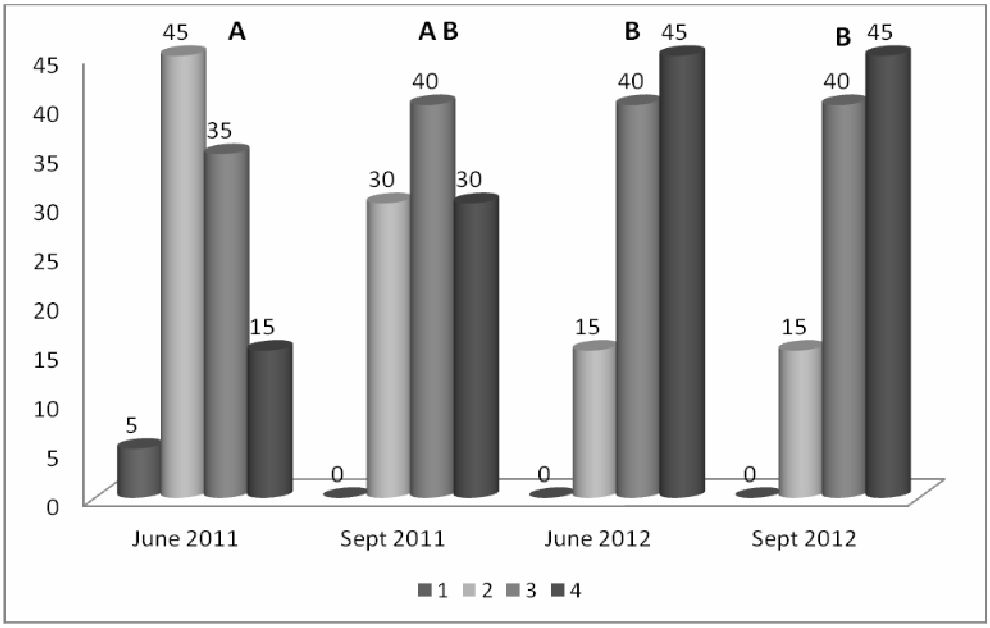

Figure 2: Changes in the health condition of the eastern black walnut trees. Categories: 1. Healthy crown; 2. Less than 20% of the crown is dying, some leaves are yellowish. 3. 20–50% of the crown is dying. Leaves with yellowish discolouration in larger groups. 4. More than 50% of the crown is dead. Yellow leaves in large groups or the remaining foliage is yellow. Values are in % of the investigated trees. The health condition of the trees in June 2011(A) is significantly different from their health condition in 2012 (B).

There was significant difference (*P = 0,009*) in the health condition of the sampling site between the investigations of June 2011 and June 2012 according to the Mann-Whitney test.

3.2 Isolation success and species composition

The isolation success was the highest in June 2011. Phytophthoras could be isolated from 75% of the collected soil samples then. After that, in September 2011 and June 2012 the isolation was succesfull only from 20% of the collected samples. The isolation success was a bit higher in September 2012. Phytophthoras could be isolated from 40% of the collected samples that time. There were two species constantly present in the soil samples: *P. plurivora* and *P. cactorum*. They were present in the rhizosphere of every selected tree, at least one time during the two – year period.

The distribution of the two species changed during the investigation (*Figure 3.*). Both species were present in June 2011 and September 2012, but only *P. plurivora* could be isolated at the other times.

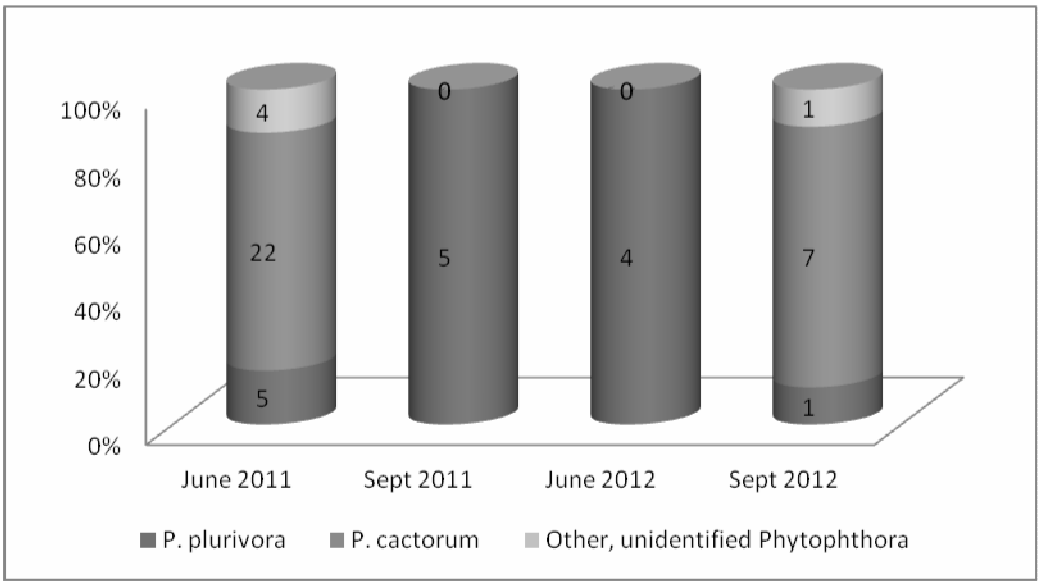

Figure 3. Species composition of Phytophthoras at different sampling times

This suggests that both species are constantly present in the soil of the forest stand. However, *P. cactorum* survives the unfavourable conditions with forming chlamydospores and oospores. When the environmental conditions become favourable, oospores and chlamydospores of *P. cactorum* can well germinate. The infection can be very serious in the case of heavy, flooded, poorly drained soils (Erwin – Ribeiro 1996).

Unlike *P. cactorum*, *P. plurivora* cannot form any chlamydospores. It can survive unfavourable environmental conditions only by forming oospores. This species is less competitive in wet, flooded soils, because supposedly semiarid – wet soils are optimal for it. However, it can infect walnut trees also under unflooded conditions (Matheron – Mircetich 1985b).

In the first year of our study, the spring and the first half of the summer was rainy. The Walter-Lieth diagram of the year 2011 (Figure 4.) shows this time as a humid period.

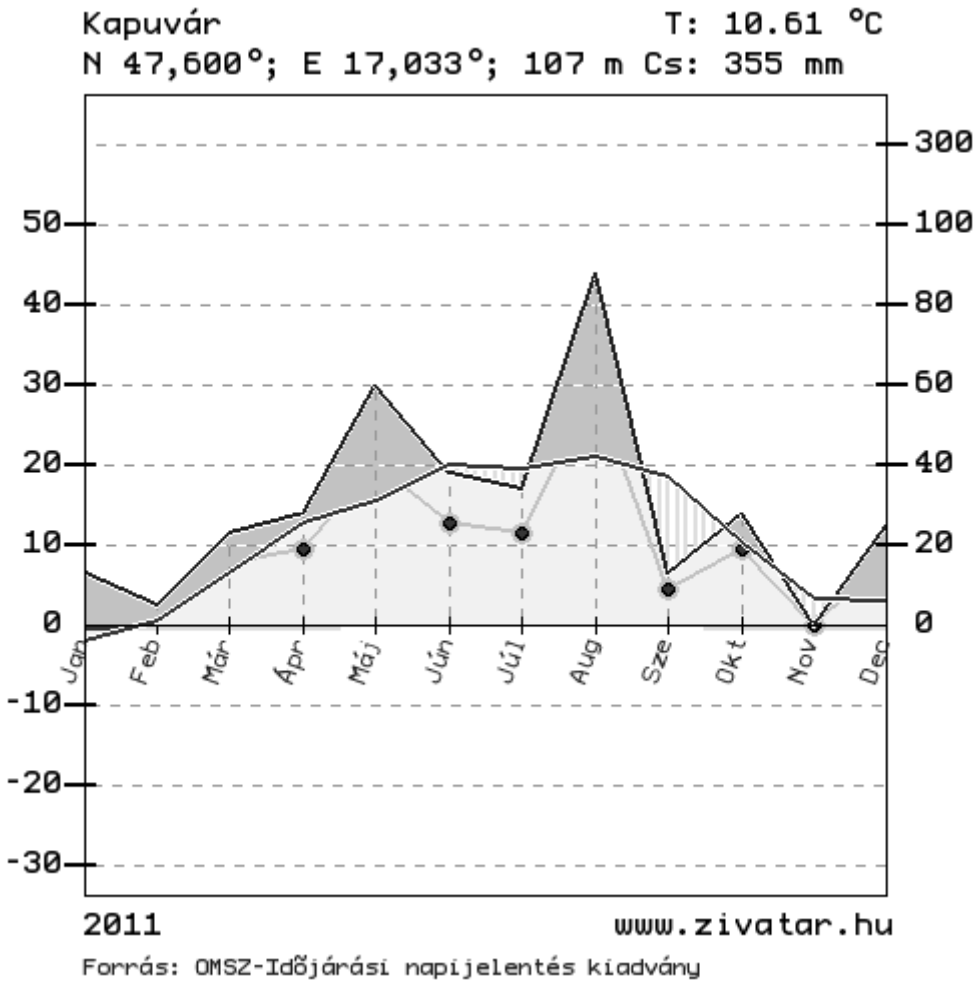

Figure 4. Walter-Lieth diagram of the sampling site in 2011 based on the data of the Hungarian Meteorological Service (http://www.met.hu/idojaras/aktualis_idojaras/napijelentes/). Dark grey: humid periods; striped: arid periods.

After 3 heavy rainfalls in the first week of August, the second half of the summer and the autumn was hot and dry, with unusual arid periods, especially in September. *P. cactorum* was dominant in June, when the forest soil was wet. Later, in mid of September, the soil was drier. This time the isolation success was lower, and we could only find *P. plurivora*.

Next year, after a dry autumn and winter in 2011, we had an extremely dry spring in 2012. According to the Walter-Lieth diagram of 2012 *(Figure 5*), the arid period began in May and lasted till September in the region.

The isolation success was low again in June, and only *P. plurivora* could be isolated. The summer was very hot, with a few big rainfall in the region mostly in the second half of July, and with some rainy days in August and September. The isolation success was a bit higher, and we could isolate mostly *P. cactorum* from the slightly wetter soil samples this time.

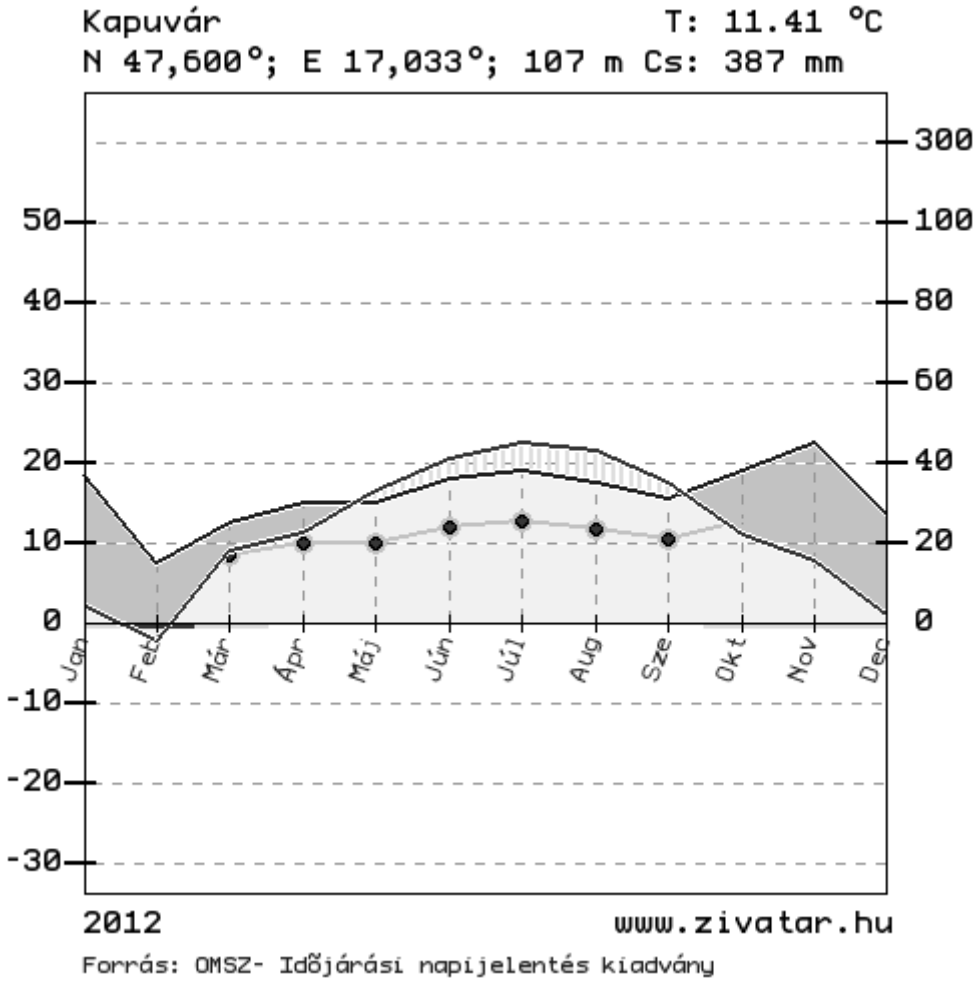

Figure 5. Walter-Lieth diagram of the sampling site in 2012 based on the data of the Hungarian Meteorological Service (http://www.met.hu/idojaras/aktualis_idojaras/napijelentes/). Dark grey: humid periods; striped: arid periods.

3.3 Intraspecific diversity

The genetic diversity of the two species was estimated based on ITS sequences. The examined fragment was 797bp long. 15 *P. cactorum* isolates and 8 *P. plurivora* isolates were analysed.

P. cactorum strains differed in 2 positions and 4 different haplotypes could be detected from which 3 haplotypes were frequent (*Table 1.*).

Table 1. Estimated haplotypes of P. cactorum *on the sampling site*

20	611	Haplotype	Number of isolates
–	A	CACTORUM 1	5
–	G	CACTORUM 2	4
A	A	CACTORUM 3	5
A	G	CACTORUM 4	1

P. plurivora strains were quite uniform, 2 haplotypes could be detected (*Table 2*). They differ only at one position. The first haplotype was much more frequent. The 2nd one is represented by only one isolate originating from the collection of the first sampling time.

Table 2. Estimated haplotypes of P. plurivora *on the sampling site*

402	Haplotype	Number of isolates
C	PLURIVORA 1	7
T	PLURIVORA 2	1

Both isolated species are cosmopolitan (Erwin – Ribeiro 1996). The poor genetic diversity especially in the case of *P. plurivora* can suggest that it might be a relatively new species in the studied region (Szabó et al. 2013).

3.4 Pathogenicity

All of the infected seedlings remained alive during the test period. Their health condition was similar as the health condition of the control plants. The stem diameter of the seedlings belonging to the control and the two infected groups was not significantly different (P = 0,107) at α=0,05 significance level according to the results of the One-Way ANOVA. However, according to the size of the necrosis on their stem, both pathogen strains turned to be moderately pathogenic to black walnut. The size of the necrosis in case of *P. plurivora* was 39,27–235,62 mm^2 (average size: 126,25 mm^2), while in case of *P. cactorum* it was 32,99-102,10 mm^2 (average: 62,93 mm^2). *P. plurivora* was slightly more aggressive than *P. cactorum*. This result agrees with the statement of Mircetich and Matheron (Mircetich – Matheron 1980). The area of the necrosis (Figure 6.) proved to be significantly larger in the two infected groups than in the case of the control seedlings at α=0,05 significance level, according to the One-Way ANOVA (P = 0,001). According to the T- test, *P. plurivora* caused significantly longer necrosis than *P. cactorum* did at α=0,05 significance level (P = 0,024).

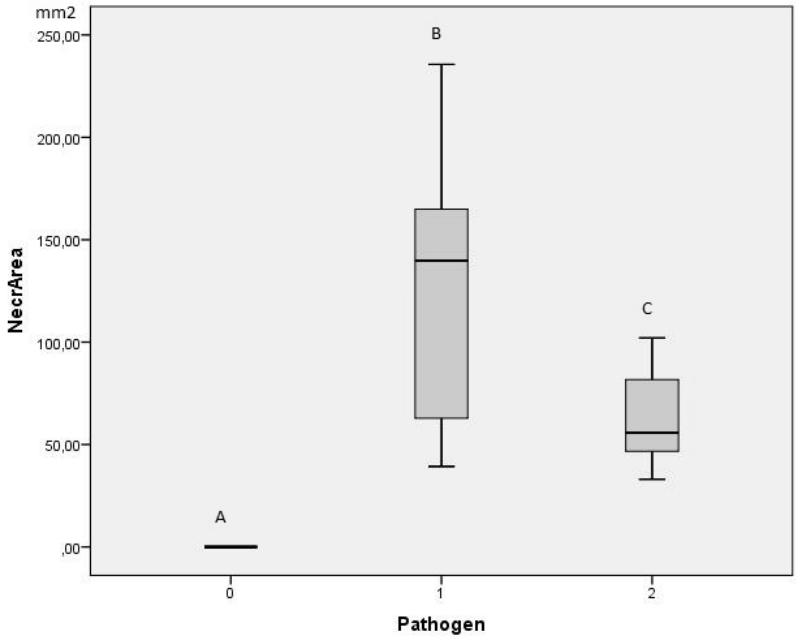

Figure 6. Necrosis area in case of the different groups. 0: seedlings without infection. 1: P. plurivora . 2: P. cactorum. The groups labelled with different letters are significantly different according to the One-Way ANOVAs and the t- test.

4. SUMMARY

Two *Phytophthora* species, *P. cactorum* and *P. pluri*vora were isolated from the rhizosphere soil of the declining old black walnut stand. These two species were also earlier frequently isolated by Szabó et al. (2013). However, we could not find *P. lacustris* as they did earlier. The health condition of the selected trees got worse during the two-years-period of the experiment. However, we couldn't find any dead trees yet. Changes could be observed in the isolation success. These changes don't show any seasonality. They could be related to the current environmental conditions, primarily with the humid or arid character of the weather conditions. The changes in the health condition of the studied eastern black walnut trees seem to confirm this. Their health condition continuously got worse in the first humid year of our investigation, while it was unaltered in the second, quite arid year. It would be necessary to continue the monitoring to find the correct correlation. Both *Phytophthora* species were isolated from the rhizosphere of every selected tree, at least at one sampling time.

The identified species composition had also changes during the two years. *P. plurivora* could be isolated always, while *P. cactorum* only in June 2011 and September 2012. When we could isolate both species, *P. cactorum* was more frequent than *P. plurivora*. We suppose that both species are present in the soil. However, *P. plurivora* is more competitive in semi-arid - wet soil, while *P. cactorum* prefers wet, flooded soil, where *P. plurivora* is less competitive. The poor genetic diversity of these species suggests that they were perhaps introduced into the region recently. Both isolated species proved to be moderately aggressive to eastern black walnut seedlings. The necroses caused by *P. plurivora* were longer than those caused by *P. cactorum*, suggesting that this species is slightly more aggressive to eastern black walnut seedlings than *P. cactorum*.

The above mentioned are preliminary results of a two-years long study. It would be necessary to do a proper analysis of weather condition data and to continue the monitoring in order to understand better the process of the decline.

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