

Post-epidemic Situation of a Previously *Phytophthora alni*-infected Common Alder Stand

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Abstract – This paper reports on the current situation of the *Phytophthora* species occurring in a declining common alder (*Alnus glutinosa*) stand in North-West Hungary. The stand was affected by a severe epidemic caused by *Phytophthora alni* in the late 1990s. The authors evaluated the health condition of the forest stand and collected soil samples from the rhizosphere of twenty selected trees two times per year in 2011 and in 2012 in order to isolate *Phytophthora* species. A diverse *Phytophthora* community was found in the soil consisting of eight species with different aggressiveness and with different ecological demands. Pathogenicity tests confirmed the role of the collected strains in the decline of the alder stand.

Root and collar rot of alder / soilborne pathogen / *Phytophthora alni* / *Phytophthora taxon Raspberry* / *Phytophthora lacustris*

Kivonat – Egy korábban *Phytophthora alni* által fertőzött mézgáséger-állomány járvány utáni állapota. A tanulmány egy pusztuló nyugat-magyarországi mézgáséger- (*Alnus glutinosa*-) állományban jelenleg előforduló *Phytophthora*-fajokról, és azok faállományra gyakorolt hatásáról tudósít, egy korábban lezajlott, *Phytophthora alni* okozta súlyos járványt követően. 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 állományban változatos, eltérő agresszivitású és környezeti igényű fajokból álló *Phytophthora* közösséget találtak. A patogenitási tesztek igazolták a gyűjtött izolátumok szerepét az erdőpusztulásban.

éger gyökér és gyökfő betegsége / talajlakó kórokozó / *Phytophthora alni* / *Phytophthora taxon Raspberry* / *Phytophthora lacustris*

1 INTRODUCTION

A severe epidemical decline of common alder (*Alnus glutinosa*) trees was observed in South-England in the summer of 1993. An unknown *Phytophthora* species was isolated from the plant tissues and rhizosphere soil samples (Brasier et al. 1995). The previously unknown ‘alder *Phytophthora*’ spread epidemically throughout Europe in the last decade of the 20th century (Cech 1998, Werres et al. 1998, Szabó et al. 2000, Jung – Blaschke 2004, Cerny et al. 2008).

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The ‘alder *Phytophthora*’ is an interspecific hybrid, a series of phenotypically diverse, heteroploid genotypes coming from asexual hybridisation (Brasier et al. 1999). The species was described in 2004 as *P. alni* and the different variants were described as subspecies: *P. alni* ssp. *alni*, *P. alni* ssp. *multiformis* and *P. alni* ssp. *uniformis* (Brasier et al. 2004). The disease can occur in various habitats and in trees representing different ages. However, the primary source of invasion might be the planting of infected saplings (Oszako 2010).

Other *Phytophthora* species were also isolated from the necrotic tissues or from the rhizosphere soil of infected alder trees. *P. citricola*, *P. cactorum*, *P. gonapodyides*, *P. megasperma* and *P. pseudosyringae* were isolated from necrotic tissues while *P. inundata*, *P. lacustris*, *P. plurivora*, *P. gregata*, *P. taxon hungarica* and *P. polonica* were isolated from rhizosphere soil (Szabó et al. 2013, Marçais – Husson 2014, Belbahri et al. 2006). These species may contribute to the decline of the alder stands (Szabó et al. 2013). However, they should not be the main reason for the epidemic alder decline (Marçais – Husson 2014).

In 1999, a severe alder decline was observed near Csorna, North-West Hungary (Szabó et al. 2000). *P. alni* ssp. *uniformis* was isolated both from rhizosphere soil samples and necrotic root tissues (Szabó et al. 2000, Nagy et al. 2003). By 2003, the health condition of the alders improved gradually (Szabó et al. 2013). Instead of *P. alni*, clade 6 *Phytophthoras* were isolated from the rhizosphere soil samples (Szabó et al. 2013). This time, alder decline was observed at several other locations in Hungary (Koltay 2007). However, in addition to *P. alni*, other *Phytophthoras* were prevalent at these sites, too (Szabó et al. 2013). In the beginning of the 21st century, frequently isolated *Phytophthora* species from alder stands were *P. gonapodyides*, *P. plurivora*, *P. inundata*, *P. megasperma*, *P. lacustris*, *P. gregata* and two undescribed taxa, *P. taxon hungarica* and *Phytophthora* sp. 1. (Szabó et al. 2013).

Although, alder decline caused by the *P. alni* subspecies is well-studied, there are still some unanswered questions, especially about the ecology and evolution of the subspecies. What happens in the surviving forest stands afterwards is also an important question.

A diverse *Phytophthora* community occurs in the soil of the alder stands because of the favourable wet conditions (Szabó et al. 2013). However, we also lack information about the impact of other *Phytophthoras* on alder trees. Monitoring the health condition and *Phytophthora*-composition in these stands is necessary to predict a potential epidemic in time.

Herein, we present the results of a post-epidemic resurvey that was carried out in an alder forest severely attacked by *P. alni*. We also report on the differential pathogenicity of various *Phytophthora* species on alder saplings as a result of two kinds of inoculation methods.

2 MATERIALS AND METHODS

2.1 Evaluation of the health condition

Twenty common alder trees were selected for sampling in a thirty-three-year-old marshland forest near Csorna, West Hungary (Figure 1). The investigated forest stands grow on fen soil with permanent water effect. The evaluation of the health condition and the sampling was done in June and September of 2011 and 2012.

Specific symptoms of *Phytophthora* root and collar rot of alders, like bark necrosis often with a dark exudate, usually appear at the base of the trunk. The health condition of trees was evaluated based on the specific collar and secondary crown symptoms. To evaluate the severity of the crown and collar symptoms, five-point scales were used (Figure 2).

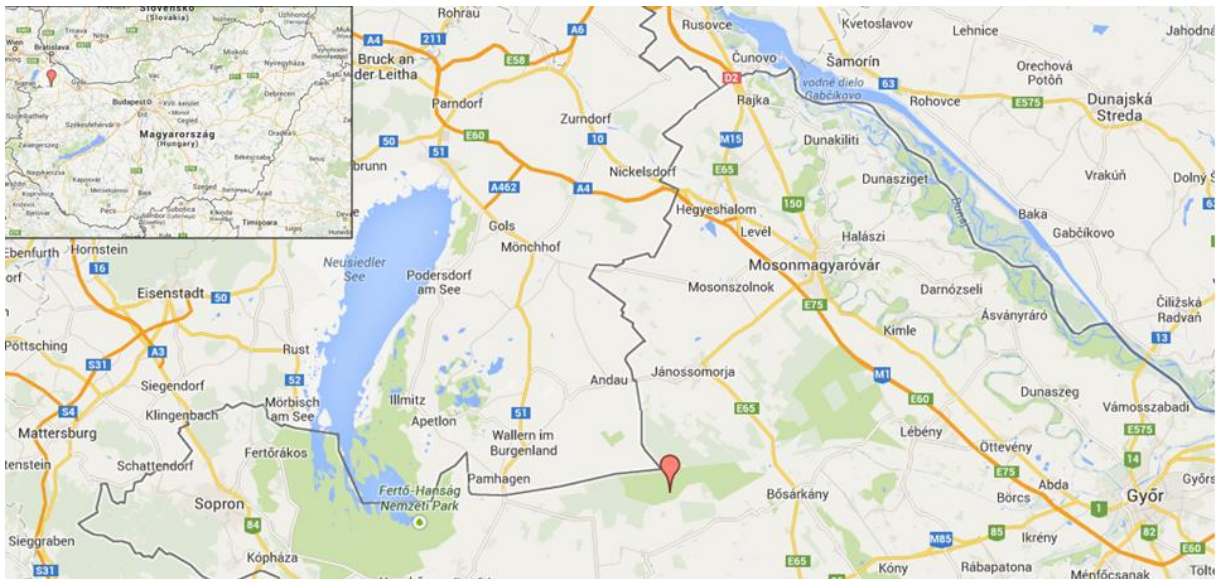


Figure 1. The sampling site. Source: Google Maps.



Figure 2. A1-A5: Health condition categories based on collar symptoms:

A1: Asymptomatic tree; A2: Bark necrosis covers less than 10% of the girth of the trunk horizontally and spreads vertically to a maximum 10% of the total height of the tree; A3: Bark necrosis covers 10–30% of the girth and height of the tree; A4: More than 30% of the total girth and height of the tree is damaged; A5: Dead tree.

B1-B5: Health condition categories based on crown symptoms:

B1: Healthy crown; B2: Less than 20% of the crown is dying. Some leaves are yellowish; B3: 20–50% of the crown is dying. Leaves with yellowish discolouration occur in groups. B4: More than 50% of the crown is dead. Yellow leaves in large groups, or the remaining foliage is yellow; B5: Dead tree.

2.2 Sampling, isolation and species identification

The method of soil sampling, sample processing and isolation were the same as reported earlier (Kovács et al. 2013). Identification of the collected isolates was based on morphological characteristics and specific molecular markers as described earlier (Kovács et al. 2013).

2.3 Pathogenicity tests

The pathogenicity of the following four most important species (collected in 2011) was tested: *P. alni*, *P. taxon Raspberry*, *P. inundata*, *P. lacustris*. Additionally, a *P. gonapodyides* strain was also tested because this species often occurred earlier in the rhizosphere soil of alders (Szabó et al. 2013). One representative isolate per *Phytophthora* species was used for inoculation. Inocula were collected from 14-day-old colonies grown on potato-dextrose agar plates (39g/L, Microtrade Ltd., Budapest, Hungary) in Petri dishes with a diameter of 90 mm.

One-year-old common alder saplings grown in plastic containers were infected. The soil for planting was tested for the lack of *Phytophthoras* using a leaf-baiting method.

Seventeen saplings per each isolate and corresponding controls (altogether 93 saplings) were wound-inoculated on April 26 and 27, 2013. A narrow wound with a length of approximately 5 mm was made on the base of the stem with a sterile scalpel and a 5 mm × 5 mm pathogen-infested agar plug was inserted in the wound. The wound was then sealed with Parafilm® (Pechiney Plastic Packaging Company). Control saplings had non-infested agar pieces in their wounds and were sealed as above. Another 17 saplings per isolate were used for root infestation (altogether 94 saplings including the control saplings). Altogether two pathogen colonies per sapling were put into the soil of the saplings at four positions around their stem.

The saplings were watered when necessary, and maintained under natural environmental conditions. After five months of incubation, disease severity of the saplings was evaluated based on five-point scales, 1 and 5 expressing no disease and lethality, respectively. In the case of the stem inoculations, the length and width [mm] of the bark lesion were measured. The average lesion size was calculated from the measured data based on the formula of an ellipsoid [mm²].

The health condition of the root system was evaluated based on a 5-point scale (Figure 3).



Figure 3: The scale for evaluating the health condition of the root system:

1. Healthy root system; 2. Root loss is below 30%; 3. 30–50% of the root system is lost;
4. Root loss is more than 50%; 5. Completely dead saplings.

The health condition of the shoots was evaluated based on a similar scale: 1. Symptomless sapling; 2. Some leaves are smaller than usual, occasionally with yellowish discolouration; 3. 30–50% of the potential crown is dead; 4. More than 50% of the potential crown is dead; 5. Completely dead saplings.

2.4 Data analysis

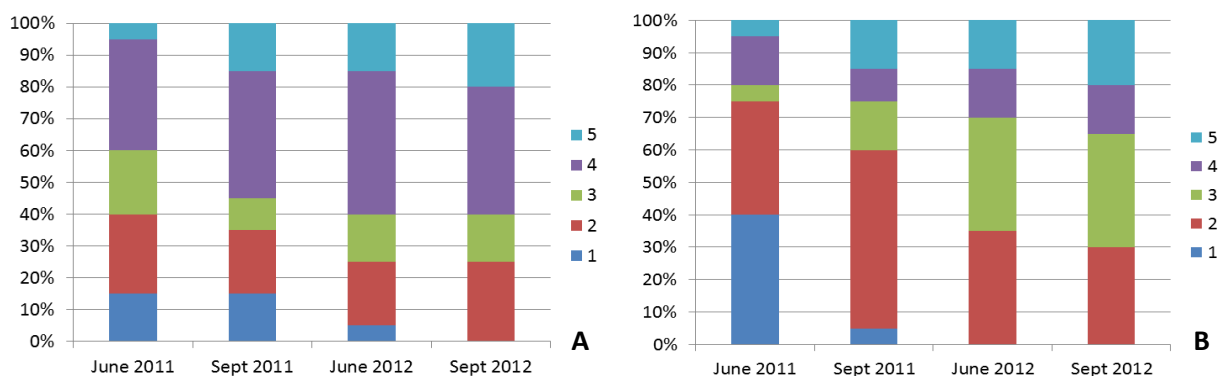
The health condition datasets were analysed with the Software STATISTICA (Ver. 11, StatSoft Inc. 2012). We used Kruskal-Wallis and Mann-Whitney tests to see the change in the health condition of our sampling site.

Data resulted from the pathogenicity tests were also analysed with STATISTICA. The Gaussian distribution of data sets was tested with the Shapiro-Wilks method. The homogeneity of variances of the variables was tested with Levene statistic, based on the median. Because the lesion size dataset does not have Gaussian distribution based on the results of the Shapiro-Wilks method, non-parametric tests were used to evaluate the pathogenicity. Kruskal-Wallis non-parametric ANOVA was used to compare multiple independent groups, and the Mann-Whitney U-test was used for pairwise comparisons of the treatment groups at $\alpha=0.05$ significance level.

3 RESULTS

3.1 Changes in the health condition

The health condition of the trees based on the bark symptoms (Figure 4A) did not change significantly during the experiment. Active lesions with fresh exudate could be observed altogether three times. Each time only one tree had fresh collar symptoms. However, the health condition of the trees based on the crown symptoms (Figure 4B) became considerably worse ($p=0.0053$) according to the Kruskal-Wallis test. Significant differences were observed between the results of June 2011 and 2012 ($p=0.0061$), and between the results obtained in June 2011 and September 2012 ($p=0.0029$). Changes are conspicuous in the decrease of the number of symptomless trees (from eight in June 2011 to zero in June and September 2012), and in the increase of the number of dead trees (from one in June 2011 to four in September 2012).



(1: best – 5: worst, details in the text)

Figure 4 A: Changes of the health condition based on the health condition categories of root collar and bark symptoms.

Figure 4 B: Changes of the health condition of the sampling site based on the health condition categories of crown symptoms.

3.2 Observed *Phytophthora* species composition

Figure 5 shows the observed species composition at different sampling times. Altogether nine *Phytophthora* taxa were isolated during the two-year survey: *P. alni* ssp. *multiformis*, *P. lacustris*, *P. taxon Raspberry*, *P. inundata*, *P. plurivora*, *P. polonica*, *Phytophthora* sp. *oaksoil*, *P. gonapodyides* and *Phytophthora* taxon *hungarica*. The most diverse species composition was observed in June 2011. Five different species, *P. alni* ssp. *multiformis*, *P. lacustris*, *P. taxon Raspberry*, *P. sp. oaksoil* and *P. inundata* were isolated at that time. Only

one species, *P. lacustris* was present at all times. *P. taxon Raspberry* was collected two times, in June 2011 and September 2012. The other species were found only once out of the four sampling times. They were present in the soil samples with low frequency.

Despite its low frequency, the presence of the highly aggressive *Phytophthora alni* in the rhizosphere soil of the trees is of great importance. At the very first sampling time, the *P. alni* ssp. *multiformis* was detected in the rhizosphere of two trees. However, at the other sampling times, we were unable to isolate this pathogen again.

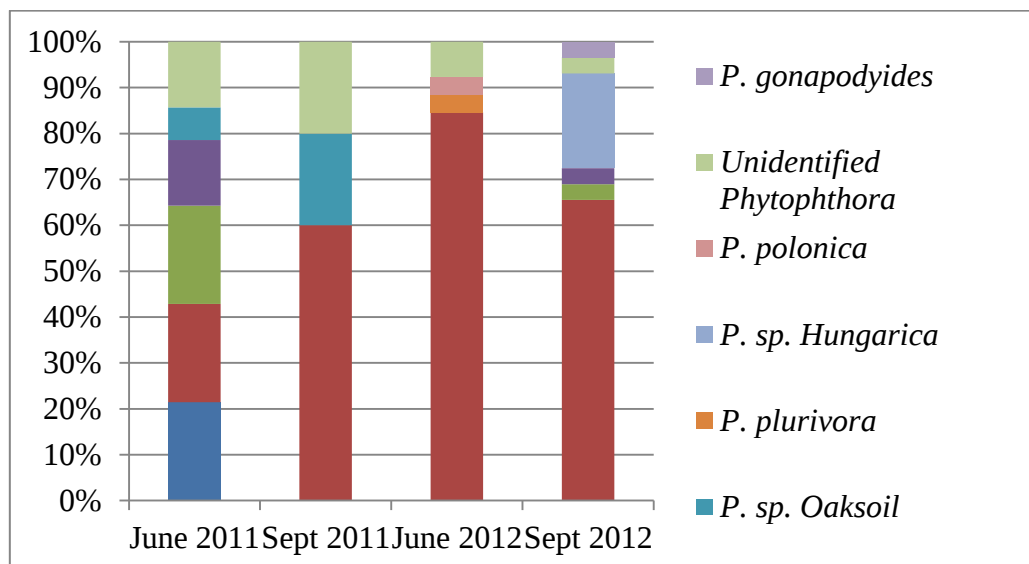


Figure 5: The observed species composition at the different sampling times.

3.3 Pathogenicity

3.3.1 Soil infestation test

Two out of 94 infected saplings died during the test period. Both saplings were infected with *P. alni* ssp. *multiformis*.

According to the results of the Kruskal-Wallis nonparametric ANOVA, the health condition of the roots of the saplings (Figure 6A) was significantly different between the groups with different treatments ($p=0.0000$). The soil infestation test did not result in significant differences in the health condition of the shoots during the test period ($p=0.0513$).

Based on the pairwise comparisons with the Mann-Whitney U-test, the health condition of the root system was worse in every infected group than it was in the non-infected control group. The most substantial damage was caused by the *P. alni* ssp. *multiformis* isolate ($p=0.0000$). Similar severe damage was caused by the *P. lacustris* ($p=0.0001$). The root system of the saplings infected with *P. taxon Raspberry* or *P. inundata* was also considerably weaker than the health condition of the roots of the control saplings ($p=0.0014$ and $p=0.0177$). However, the root system of *P. gonapodyides*-infected saplings was not significantly damaged in comparison with the root system of control saplings ($p=0.1497$). *P. alni* damaged the root system more severely than *P. taxon Raspberry* ($p=0.0293$), *P. inundata* ($p=0.0002$) or *P. gonapodyides* ($p=0.0001$). Damage caused by *P. inundata* was milder than that caused by *P. lacustris* ($p=0.0020$), but was not much different from the damage caused by *P. gonapodyides* ($p=0.4745$). *P. lacustris* caused significantly more severe damage to alder roots, than *P. gonapodyides* did ($p=0.0006$). The damage caused by *P. taxon Raspberry* did not differ greatly from the damage caused by *P. inundata* ($p=0.1729$), *P. lacustris* ($p=0.1434$) or *P. gonapodyides* ($p=0.0665$).

3.3.2 Stem inoculation test

Four out of 93 saplings, i.e. those inoculated with the *P. alni* ssp. *multiformis* isolate, died during the test period.

According to the results of the Kruskal-Wallis non-parametric ANOVA, the treatment groups differ to a great extent in terms of the health condition of the shoots of saplings ($p=0.0011$). As to the condition of the root system of saplings, there are also pronounced differences between the groups ($p=0.0007$), albeit the planting media of these saplings was not infected with *Phytophthora*. The lesion sizes (Figure 6. B) were also significantly different ($p=0.0000$) between the treatment groups.

Based on the pairwise comparisons with the Mann-Whitney U-test, the health condition of the shoots was the poorest in the *P. alni*-infected group. It was considerably poorer than in the non-infected control saplings ($p=0.0244$), as well as in the *P. taxon Raspberry*-infected ($p=0.0081$), the *P. gonapodyides*-infected ($p=0.0027$) and the *P. inundata*-infected ($p=0.0114$) groups.

However, based on the symptoms of the *P. alni*-infected group, the health condition of the saplings did not differ significantly from the health condition of the *P. lacustris*-infected group ($p=0.5228$). The health condition of the shoots in the *P. lacustris*-infected group were damaged much more than the shoots in the *P. taxon Raspberry* ($p=0.0380$), *P. gonapodyides* ($p=0.0131$) and *P. inundata*-infected ($p=0.0487$) groups.

Although only wound inoculation was carried out, there were notable differences in the health condition of the roots between the treatment groups. Based on the health condition of the root system, the most severe damage was caused by the *P. alni* ssp. *multiformis* isolate. The root systems of the saplings in the *P. alni* ssp. *multiformis*-inoculated group were significantly more degraded than the roots of the control saplings ($p=0.0114$), the *P. taxon Raspberry*-infected ($p=0.0401$) saplings and the *P. inundata*-infected ($p=0.0309$) saplings. Furthermore, *P. lacustris* caused substantial damage to the roots compared to the control saplings ($p=0.0177$). However, its impact was not significantly different from that of the other three *Phytophthora*s used for the inoculation test. *P. alni* ssp. *multiformis* and *P. lacustris* seem to be able to colonize roots secondary through the transport vessels of the sapling.

Every *Phytophthora* species used for the stem inoculation proved to be pathogenic to alder saplings ($p=0.0001$). The minimum, average, and maximum area of the lesions caused by the isolates used are summarized in Table 1. The lesions caused by the *P. alni* ssp. *multiformis* strain were considerably larger than those caused by *P. lacustris* ($p=0.0309$), *P. taxon Raspberry* ($p=0.0031$), *P. gonapodyides* ($p=0.0031$) and *P. inundata* ($p=0.0008$). The lesions caused by *P. lacustris* were also ascertainably larger than those caused by *P. taxon Raspberry* ($p=0.0027$), *P. gonapodyides* ($p=0.0040$) and *P. inundata* ($p=0.0001$).

Table 1: Average size of lesion caused by the species used for the wound inoculation experiment

Species	Area of lesion (mm ²)		
	minimum	average	maximum
<i>P. taxon Raspberry</i>	7.85	56.51	188.50
<i>P. alni</i> ssp. <i>multiformis</i>	0.00	502.32	1350.90
<i>P. gonapodyides</i>	0.00	45.61	
<i>P. inundata</i>	0.00	33.68	66.76
<i>P. lacustris</i>	31.42	132.59	522.29
Control	0.00	0.00	0.00

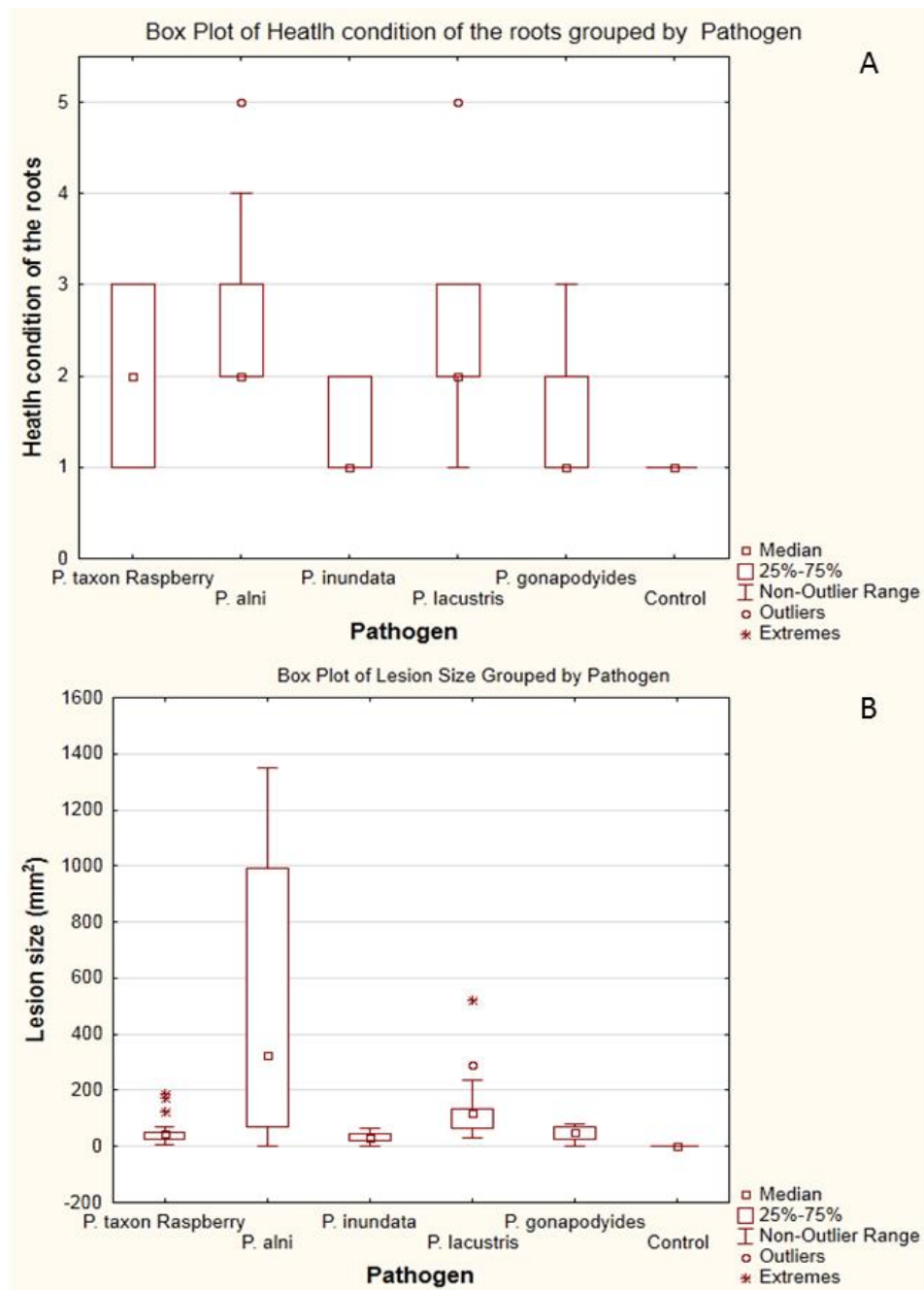


Figure 6: A: Health condition of the roots (soil infestation). B: Average size of the stem lesions (mm²) caused by the different pathogens.

4 DISCUSSION

The collar symptoms of alder trees observed at the sampling site predominantly included cankers and tarry spots remaining from an earlier *Phytophthora alni*-infection. Active collar symptoms with fresh exudates were observed altogether on three different trees, at one sampling time each. This explains that the health condition of the trees seems to stagnate based on the dataset referring to collar symptoms. The observed crown symptoms are secondary symptoms ensuing from a potential root infection. The negative tendency of the health condition based on the crown symptoms, and the increasing number of dead trees may be in correlation with the *Phytophthora* species found in the rhizosphere of the investigated trees.

Both morphological and molecular methods were used to identify the collected isolates. Changes in the local composition of *Phytophthora* species were observed during the monitoring period. Weather extremities and changes in the soil moisture content may correlate stronger than seasonality to the observed alterations in species composition. Regarding *P. alni*, it has been isolated from the rhizosphere of two trees only, although it was not present in the soil between 2003 and 2008 (Szabó et al. 2013). In recent studies, we have isolated *P. alni* ssp. *multiformis*, whereas before 2003 *P. alni* ssp. *uniformis* was present in the stand. Based on the study of Marcais – Husson (2014), the presence of *P. alni* suggests that all trees on the studied site might again be attacked by *P. alni*. Conclusively, completely symptomless alder trees might harbour *P. alni* in their root system and as such they become the main inoculum source (Elegbede et al. 2010). Other *Phytophthoras*, like those in clade 6, are consistently dominant in forest soil with the permanent presence of water as was the case with our sampling area. However, *P. gonapodyides*, a species which had been multitudinous, was only once isolated during recent surveys while *P. lacustris* became the most frequently isolated *Phytophthora*. *P. lacustris* seems to be significantly more aggressive than *P. gonapodyides* on common alder. The new appearance of *P. alni* and the continuous presence of *P. lacustris* may explain the considerable condition decline based on crown symptoms of the trees in 2011.

The *Phytophthora* species chosen for the pathogenicity tests are soil-borne pathogens. They primarily damage the root system. Symptoms in the aboveground parts usually appear with a delay. This is why the soil infestation tests showed no significant differences in the health condition of the shoots, but they did reveal significant differences in condition of the root system between the treatment groups.

Among the studied *Phytophthoras*, the aggressive *P. alni* is able to infect trees through wounds or lenticels of the trunk. In this case, it damages the transport vessels in the roots and stem. The change of water and assimilates fails between the roots and the crown. The process leads to the death of the tree (Obwald et al. 2014). This may explain the decline of root conditions in the *P. alni*-infected group in comparison with the other groups by the wound-inoculation test.

In a pathogenicity test Szabó and Lakatos (2008) used only the stem inoculation method on alder saplings with one isolate each of *P. gonapodyides*, *P. alni* ssp. *uniformis*, *P. inundata* and a then unknown taxon similar to *P. lacustris*. The average lesion size caused by that *P. gonapodyides* isolate was more than two times larger (93.49 mm^2) than the lesions caused by our isolate. This suggests quite a high intraspecific variation in aggressivity. Our *P. alni* ssp. *multiformis* isolate caused lesions that were almost three times larger than the ones caused by *P. alni* ssp. *uniformis* isolate (172.79 mm^2). The average lesion size caused by their *P. inundata* isolate (36.76 mm^2) is comparable to ours. Brasier and Kirk (2001) used, among other *Phytophthora* spp., *P. alni* and *P. gonapodyides* isolates for wound inoculations. In accordance with our results, they found that *P. alni* was the most aggressive *Phytophthora* species on common alder, while lesions caused by *P. gonapodyides* did not differ considerably from those on the control logs. Furthermore, Nechwatal et al. (2013) carried out both stem inoculations and soil infestations. In the case of root infection, both *P. gonapodyides* and *P. lacustris* significantly reduced the total dry mass of the roots of common alder saplings (Nechwatal et al. 2013). Both species caused similarly small lesions. The lesions did not differ much from those on the non-infected control saplings, while they were significantly smaller than the lesions caused by *P. alni* in their case study (Nechwatal et al. 2013). Our results with *P. gonapodyides* are well matched with their results, but with *P. lacustris*, our results suggest a higher intraspecific variability in pathogenicity.

P. inundata seems primarily to be a parasite of riparian woody species but also of some tree species like *Juglans regia* or *Prunus* spp. under horticultural circumstances (Brasier et al.

2003). In forest ecosystems, it was affiliated with root rot of olive (*Olea europea*) and species of the genera *Fagus*, *Castanea*, *Salix* and *Alnus* (Brasier et al. 2003, Safaiefarahani et al. 2013, Szabó – Lakatos 2013). It may cause the death of the host trees after heavy rainfalls and floods (Brasier et al. 2003). Therefore, after the extremely wet year of 2010, it is not surprising that we found *P. inundata* at our very first sampling.

P. taxon Raspberry is a clade 6 taxon, like its close relatives *P. lacustris*, *P. gonapodyides* and *P. inundata*. Data on pathogenicity tests with this species, however, are not available. Still, it is suggested that it may be an opportunistic pathogen similar to the other clade 6 species (Jung et al. 2011). The results of our pathogenicity tests support this hypothesis.

5 SUMMARY

A diverse *Phytophthora* community was present in the rhizosphere of the tested common alder trees. Of the eight species we found, only *P. lacustris* was isolated continuously. *P. sp. 'oaksoil'* and *P. taxon Raspberry* were found twice, while the other five species were only found once each. The highly aggressive *P. alni* was detected at the site again after more than ten years. At the very first sampling time, *P. alni* ssp. *multiformis* was isolated from the rhizosphere of two trees. However, earlier *P. alni* ssp. *uniformis* caused epidemic in the stand. Instead of *P. gonapodyides*, the more aggressive *P. lacustris* became the dominant *Phytophthora* species. *P. inundata*, *P. plurivora* and *P. taxon hungarica* were present in the soil, as earlier. However, instead of *P. megasperma*, *P. polonica* and *P. sp. 'oaksoil'* could be isolated.

Although there was no *Phytophthora*-epidemy observed at our representative sampling site, the decline of the trees was significant. Active collar symptoms were observed only in a few cases, while the number of dead trees increased and the health condition of the trees decreased, especially in the first year of our study.

Both pathogenicity tests confirmed the different pathogenicity of the isolated *Phytophthoras* on common alder saplings. Based on our results, the most aggressive *Phytophthora* pathogen of common alder is *P. alni*, while the second most aggressive is *P. lacustris*, followed by *P. taxon Raspberry* and *P. inundata*. *P. gonapodyides* was not confirmed to be pathogenic to alder roots. However, with its small lesions it proved to be slightly pathogenic to common alder saplings upon wound inoculations. Our results suggest that the appearance of *P. alni* and the continuous presence of *P. lacustris* may correlate with the decline of alders.

Our pathogenicity tests conclusively demonstrate the different pathogenicity of *Phytophthora* spp. on young alder saplings. However, their aggressiveness might be weaker on mature trees, thus the development of decline may be long-continued.

These results are based on a two-year-long field experiment, conducted in a representative sampling site of a marshland common alder forest. The changes observed during the survey period might also be true for similar forest stands surrounding the sampling site.

In order to fully understand the epidemiological and ecological aspects of alder decline, it might be necessary to correlate the abiotic environmental conditions of the sampling site with the observed results.

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