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Influence of low temperature and frost duration on *Phytophthora alni* subsp. alni viability

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Abstract

Limits on the survival of *P. alni* subsp. *alni* (PAA) due to low temperature can be expected based on previously published laboratory and field studies. This study presents a laboratory experiment to test the influence of low temperature and frost duration on PAA viability. Ten PAA isolates were incubated at different temperatures (–0.1, –2.5, –5.0, –7.5, and –10.0 °C) and frost durations (0 – 7, 14, 21, and 28 days). A regression analysis confirmed the significant influence of both factors (low temperature and frost duration, and their interaction) on the survival of the pathogen under laboratory conditions. The survival and failure time analysis showed that the survival of the pathogen differs significantly after mild frost (all the isolates tested survived temperatures between –0.1 and –5.0 °C during the entire testing period) and heavy frost (the pathogen died after 21 days of incubation at –7.5 °C and after 2 days at –10.0 °C). Moreover, the viability of the pathogen decreased significantly if the temperature of –5.0 °C was maintained for at least 1 week and the temperature of –7.5 °C persisted in laboratory conditions for at least 4 days. The results of the study proved the pathogen to be very sensitive to heavy frost. The low-temperature limits for PAA occur regularly in Central Europe in January. It is probable that these temperatures can reduce PAA populations in diseased black alder stems. The climate change characterised by increases in the lowest minimum winter temperatures in Central Europe (as hypothesised by IPCC) may pose a significant risk for affected alder population in the area.

Key words: *phytophthora* alder disease; winter survival; viability; temperature; frost.

Resumen

Influencia de las bajas temperaturas y la duración de las heladas en la viabilidad de *Phytophthora alni* subsp. alni

Se pueden esperar límites a la supervivencia de *P. alni* subsp. *alni* (PAA), debido a las bajas temperaturas basándose en resultados obtenidos en laboratorio y estudios de campo. Este estudio presenta un experimento de laboratorio para probar la influencia de las bajas temperaturas y la duración de las heladas sobre la viabilidad de PAA. Se incubaron diez cepas PAA a diferentes temperaturas (–0,1, –2,5, –5,0, –7,5 y –10,0 °C) y duración de heladas (0 – 7, 14, 21, y 28 días). Un análisis de regresión confirmó la importante influencia de ambos factores (bajas temperaturas y la duración de heladas, así como su interacción) en la supervivencia del patógeno en las condiciones de laboratorio. La supervivencia y el análisis de tiempo de fallo demostró que la supervivencia del patógeno difiere significativamente después de las heladas suaves (todas las cepas aisladas sobrevivieron a temperaturas entre –0,1 y –5,0 °C durante el período completo de la prueba) y heladas fuertes (el patógeno murió tras 21 días de incubación a –7,5 °C y después de 2 días a –10,0 °C). Además, la viabilidad del patógeno disminuyó significativamente si la temperatura de –5,0 °C se mantenía durante al menos 1 semana, o la temperatura de –7,5 °C se mantenía durante al menos 4 días. Los resultados del estudio muestran que el patógeno es muy sensible a las heladas intensas. Los límites de baja temperatura para el PAA se producen regularmente en Europa Central en enero. Es probable que estas temperaturas puedan reducir las poblaciones de la PAA en los árboles enfermos de aliso negro. El cambio climático caracterizado por el aumento de las temperaturas mínimas más bajas del invierno en Europa Central (como hipotetiza el IPCC) puede suponer un riesgo significativo para la población afectada de alisos en este área.

Palabras clave: *Phytophthora* de alisos; supervivencia invernal; viabilidad; temperatura; helada.

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Introduction

The alder pathogen *Phytophthora alni* subsp. *alni* Brasier et S.A. Kirk (PAA) causes devastating epidemics in black and grey alder in many European countries (Gibbs *et al*., 1999; Jung and Blaschke, 2004, 2006; Schumacher, 2006; Streito *et al*., 2002), including the Czech Republic (Černý and Strnadová, 2010). The pathogen causes lethal root and collar rot on the hosts and can persist in the host tissues for several years. In naturally regenerating riparian alders, the infection develops especially at the collar or, less frequently, on the surface of exposed large roots (Jung and Blaschke, 2004). The impact of *Phytophthora* pathogens on their hosts can be affected by the amount of surviving inoculum (Erwin and Ribeiro, 1996). Based on field observations, laboratory experiments (Schumacher *et al*., 2006), and ecological modelling (Downing *et al*., 2008) the pathogen is supposed to be sensible to low temperature. It is probable that the amount of surviving inoculum will increase as the temperatures increase during the winter (Bergot *et al*., 2004; Coakley and Scherm, 1996). The successful survival of the black alder pathogen can be affected by the winter temperature for several reasons: (1) the absence of chlamydospores as potential resting structures (Brasier *et al*. 2004); (2) the failure of oospore germination connected with meiotic irregularities in this hybrid (Brasier *et al*., 2004; Delcán and Brasier, 2001); (3) the reported poor survival ability of the pathogen in the soil and the rhizosphere (Delcán and Brasier, 2001; Jung and Blaschke, 2004).

The aim of this study is to verify the sensitivity of the pathogen to frost by observing *in vitro* incubation.

Material and Methods

Ten PAA isolates were isolated from active stem lesions of *Alnus glutinosa* trees (Černý and Strnadová, 2010) growing in riparian stands in different areas of the Czech Republic from 2006 through 2008 and preserved in culture collection of the Silva Tarouca research institute in tubes on oatmeal agar (50 g oatmeal, 15 g agar per litre of deionised water) under mineral oil at approximately 12 °C (Table 1). The identity of isolates to PAA was confirmed using the allele specific primers of, *ASF*-like, *GPA*1, and *TRP*1 gene regions (Ioos *et al*. 2006).

Table 1. The list of *P. alni* subsp. *alni* isolates used in the experiment and climate characteristics of their locations (the data about climatic region and January temperature adopted from Tolasz *et al.,* 2007)

No of isolate	Location	Year of isolation	Climatic region	January temperature (°C)
P028.06	Heřmaničky (northern Bohemia)	2006	MW9	–2 ; –1
P061.07	Čakovice (central Bohemia)	2006	MW5	–2 ; –1
P135.07	Varvažov (southern Bohemia)	2007	MW11	–2 ; –1
P136.07	Horšovský Týn (western Bohemia)	2007	MW10	–3 ; –2
P137.07	Sedlčany (central Bohemia)	2007	MW10	–2 ; –1
P146.07	Jenišov (western Bohemia)	2007	MW4	–3 ; –2
P195.07	Klikov (southern Bohemia)	2007	MW4	–3 ; –2
P199.07	Sojovice (central Bohemia)	2007	W2	–2 ; –1
P221.08	Trhové Sviny (southern Bohemia)	2008	MW11	–3 ; –2
P223.08	Hamr (southern Bohemia)	2008	MW5	–3 ; –2

The isolates were refreshed and subsequently cultivated on carrot agar plates (CA; 50 g sliced carrot and 15 g agar per litre of deionised water; 25 ml medium per Petri dish) at 20 °C in the dark. Agar plugs (0.5 cm diam.) with well-developed oospores were taken from the margins of two-week-old colonies. The plugs were then placed, mycelium downwards and at regular intervals, on V8 juice agar plates (V8A; 100 ml V8 juice, 15 g agar, and 3 g $CaCO_3$ only per litre of deionised water; 25 ml per Petri dish) and subsequently incubated in the thermostat. The sensitivity of the pathogen to frost was tested over a range of temperatures (–0.1, –2.5, –5.0, –7.5, and –10.0 °C) and frost durations (0, 7, 14, 21, and 28 days). The sensitivity to the lowest temperatures between –5.0 and –10.0 °C was also tested after 1 to 7 days because of the expected rapid effects of heavy frost.

The viability of the pathogen was tested with incubation of ten CA plugs of particular isolates on individual V8A plates in each treatment combination (isolate × temperature × frost duration) in the dark. After exposure to the frost, the V8A plates with plugs were incubated at 20 °C in the dark. The number of colonies emerging around the plugs (surviving particles) were counted after 7-21 days on each V8A plate. The V8A plates inoculated in the same way and incubated at 20 °C in the dark served as a control.

The software package Statistica 7.0 (StatSoft, Inc., Tulsa, OK) was used for statistical analysis. The data were processed with non-parametric survival and failure time analysis. If post-hoc tests were needed, the data were square-root transformed. If the normality and homogeneity assumptions were satisfied, a Tukey post-hoc test was used. In other cases, the data were analysed with a non-parametric Kruskal-Wallis test. The data were also processed with general regression models (GRMs). Because certain data groups did not have a normal distribution, the data were rank transformed and then tested with regression analysis (Bonate, 2000).

Results

The Gehan-Wilcoxon test showed that the PAA survival and failure time varied significantly and clearly depended on the tested values of temperature between –0.01 and –10.0 °C ($p < 0.001$; Figure 1). Differences among incubation at –0.01, –2.5, and –5.0 °C were not found ($p > 0.05$) because all the isolates survived over the course of the entire experiment.

At –0.1 °C and –2.5 °C, all tested isolates survived the entire test period (28 days) without a decrease in viability because all inoculated CA plugs given pathogen colonies at the end of the experiment. However, the incubation at –5.0 °C showed a substantial decrease in the viability of the pathogen after 7 days ($p < 0.01$; Tukey post-hoc test), and all isolates survived the experiment at frequencies of approximately 10 – 20% (Table 2).

The Gehan-Wilcoxon test showed that survival at –7.5 and –10.0 °C differed significantly from survival at the three other temperatures tested ($p < 0.001$). All the PAA isolates tested died before the end of the ex-

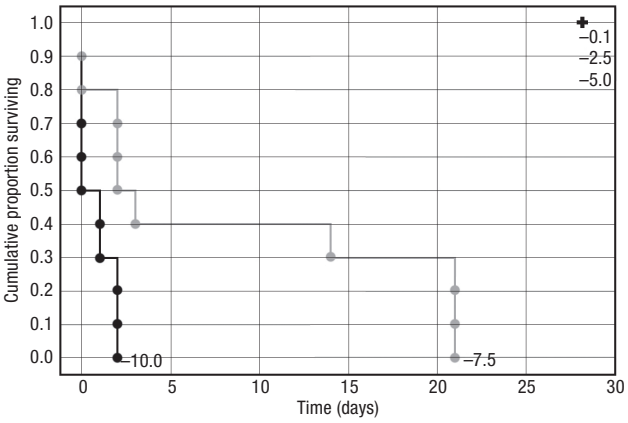

Figure 1. Kaplan-Meier graph of cumulative proportion surviving of *P. alni* subsp. *alni* isolates during incubation at temperatures –0.01, –2.5, –5.0, –7.5, and –10.0 °C.

Table 2. Summary of survival and failure of *P. alni* subsp. *alni* during the incubation at temperatures between –0.01 and –10.0 °C. *non-parametric Kruskal-Wallis test, S.D.: standard deviation

Temperature (°C)	Significant ($p < 0.01$) decrease of viability (days)	50% failure (days)	Failure (days)	Survival after 28 days (%, S.D.)
–0.1	–	–	–	100 (±0.00)
–2.5	–	–	–	100 (± 0.00)
–5.0	7	–	–	0.12 (±0.04)
–7.5	4*	2	21	0 (±0.00)
–10.0	3*	1	2	0 (±0.00)

periment (Figure 1). The difference between the values of survival at the two lowest temperatures was smaller ($p < 0.05$). The temperature of –7.5 °C rapidly reduced the viability of the pathogen. A significant decrease in viability occurred after 4 days of incubation ($p < 0.01$, Kruskal-Wallis test). The 50% failure level occurred after 2 days of incubation at –7.5 °C. Only three isolates survived for 3 weeks at very low frequencies. No isolate survived after 28 days of incubation. The temperature –10.0 °C had a very rapid effect on pathogen survival. A failure level of 50% occurred after 1 day of incubation, and no isolate survived for 3 days at that temperature (Figure 1, Table 2). The influence of the temperature (especially the low temperatures –7.5 and – 10.0 °C) on survival is apparent from the graph of the regression (Figure 2).

A significant GRM was obtained ($F = 359.26$, $p < 0.01$). Temperature, frost duration, and their interaction were significant in this model (Table 3). The coefficients of the significant factors were as follows: temperature ($\beta = 0.54$); frost duration ($\beta = -0.63$); and the temperature × frost duration interaction ($\beta = 0.45$).

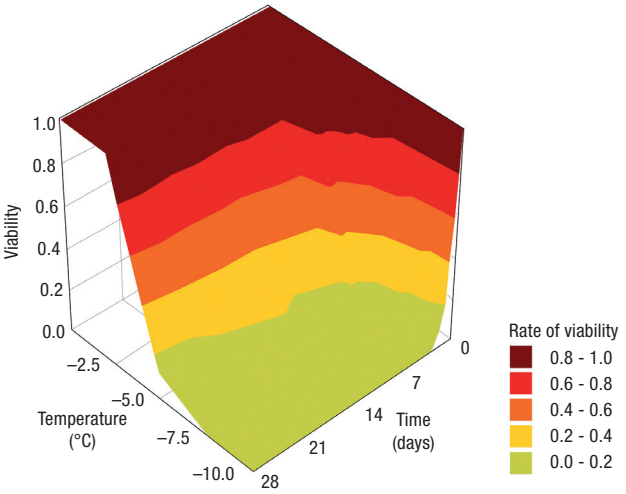

Figure 2. The graph of regression of *P. alni* subsp. *alni* survival on temperature and frost duration. Viability levels are highlighted.

Discussion

The sensitivity of the pathogen to heavy frost was confirmed by this *in vitro* experiment. This finding corresponded to the hypothesised effects of heavy frost based on the absence of potential survival structures in PAA, the low competitive ability of the pathogen, and the sensitivity of the pathogen to frost in the field (Brasier *et al*., 2004; Delcán and Brasier, 2001; Jung and Blaschke, 2004; Schumacher *et al*., 2006). However, the temperatures here tested are just those affecting the mycelium and it is also probable that the pathogen can escape the influence of heavy frost by overwintering in roots in non-frozen layers of soil or water.

The relative tolerance of PAA to mild frost (up to –2.5 °C without a significant decrease in viability) is consistent with the distribution of the pathogen in areas with cold continental winters, such as Sweden, Estonia, Lithuania, Poland, and the Czech Republic (Černý and Strnadová, 2010; Jung and Blaschke, 2004; Oszako and Orlikowski, 2005). The finding of mild frost tolerance also agrees with the relative cold tolerance of the pathogen shown by its lower cardinal temperatures and previously reported survival at 0 °C (Brasier *et al*., 1995, 2004; Schumacher *et al*., 2006).

The finding that PAA can effectively survive mild frost but cannot survive heavy frost can be considered in light of the outcomes of the field study (Černý and Strnadová, accepted) in combination with known climatological data. The pathogen was found to survive successfully in 86.09% of the samples after the mild winter of 2006/7 with the average temperature was 2.54 °C (data from Czech Hydrometeorological Institute). In contrast, the survival of the pathogen was poor (only 13.91% of the samples) following the typical winter of 2008/9 (the average temperature was –1.96 °C). It is probable that the difference in pathogen survival between winters was

Table 3. General regression model summary for dependent variable (viability of *P. alni* subsp. *alni* isolates). SS: sum of squares, d.f.: degrees of freedom, MS: mean square, F: F-ratio, *p*: *p*-value

	SS	d.f.	MS	F	*p*
Intercept	777,970	1	777,969.9	204.423	< 0.001
Temperature	392,856	1	392,855.8	103.229	< 0.001
Frost duration	511,397	1	511,396.8	134.377	< 0.001
Temperature × Frost duration	129,488	1	129,487.7	34.025	< 0.001
Error	1,621,225	426	3,805.7		

connected with the occurrence of heavy frost but not with the average winter temperature. It was found that during the mild winter, only 3 days with an average temperature of –5 °C or below occurred in the study area. Moreover, temperatures continuously below –5 °C lasted fewer than 24 hours, and only 2 decreases of temperature to values below –10.0 °C were recorded. During the typical winter, 23 days with average temperatures below –5 °C occurred. The longest period with temperatures continuously below this value was 4 days in length. Moreover, 10 temperature decreases to values below –10.0 °C were recorded. If the pathogen failure is determined by heavy frost as expected, the climate change characterised by an increase in the lowest minimum winter temperatures expected in Central Europe (IPCC, 2007) may pose an important risk to the affected host populations in the area and also its spreading in latitude and altitude.

A potential adaptation of PAA to the colder climates of northern latitudes or higher altitudes is possible, because the pathogen has occurred in Europe since the 1980s (Jung and Blaschke, 2004) and currently occupies an extensive area from Ireland to Slovenia (Jung and Blaschke, 2004; Munda *et al*., 2006). Moreover, the pathogen probably occupies a broad range of altitudes – in the Czech Republic, for example, it was isolated between 174-793 m a.s.l (Černý and Strnadová, unpubl.). The possible existence of variability in PAA sensitivity to frost may be supported by the fact that two isolates (P 135.07 and P 199.07) were among the three most successfully surviving isolates at the two coldest temperatures (–7.5 °C and –10.0 °C). The solution of this problem requires considerably more extensive experimentation.

Conclusion

The influence of temperature and frost duration on the viability of PAA isolates *in vitro* was experimentally confirmed. The temperatures –0.1 and –2.5 °C did not have a significant effect on the viability although the incubation lasted for 4 weeks. The viability of the pathogen decreased significantly if the temperature of –5.0 °C persisted for at least 1 week and if the temperature of –7.5 °C persisted for 4 days. It was found that heavy frost has a strong influence on the failure of PAA. All tested isolates died within 2 days of incubation at –10.0 °C. The climate change characterised by increases in the lowest minimum winter temperatures in Central Europe (as hypothesised by IPCC) may pose an important risk to further spreading of disease on host population in the area. The ultimate solution to the problem of PAA winter survival requires additional experimentation involving the incubation of inoculated host tissues *in vitro* and *in vivo*.

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