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# Evaluation of the Susceptibility of Several Czech Conifer Provenances to *Fusarium circinatum*

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**Abstract:** Pine pitch canker (PPC), caused by *Fusarium circinatum*, is considered among the most important diseases affecting pines in many locations throughout the world. In Europe, *F. circinatum* is currently present in the Iberian Peninsula, posing a high risk of its spread into currently disease-free countries in Europe. In the present study, the susceptibility of *Pinus sylvestris*, *Picea abies*, and *Larix decidua* originating in the Czech Republic to *F. circinatum* was tested. Furthermore, the presence of asymptomatic yet infected seedlings was also checked. This study demonstrated the pathogenicity of *F. circinatum* to the Czech provenance of *P. sylvestris*, whereas *Picea abies* and *Larix decidua* proved to be tolerant. The reisolation of *F. circinatum* beyond the inoculation point demonstrated that this quarantine pathogen is able to infect the three conifers tested, giving rise to asymptomatic seedlings for at least eight and a half months. To our knowledge, this is the first study in which the presence of symptomless seedlings has been recorded in the genera *Picea* and *Larix*. This finding points out that the European legislation would fail to avoid the risk of new introductions via symptomless seedlings, since this legislation is only restricted to plants of the genus *Pinus* and the species *Pseudotsuga menziesii*.

**Keywords:** pitch canker; provenance; pathogenicity; symptomless; *Pinus*; *Picea*; *Larix*

## 1. Introduction

Forests are important ecosystems from both ecological and economic perspectives. Approximately 33.85% (2,669,850 hectares) of the Czech Republic is aforested, of which 72.1% are conifer forests [1]. Among the conifers, Norway spruce (*Picea abies* L.), Scots pine (*Pinus sylvestris* L.), and to a lesser extent, European larch (*Larix decidua* Mill.) are the most important species. In fact, *P. abies* and *P. sylvestris* are native species covering 1,312,204 and 425,687 ha, respectively (i.e., 50.5% and 16.4% of the total forest area in the Czech Republic) [1].

Nowadays, invasive alien species are a major threat to forest ecosystems. In particular, forest pathogens introduced into countries as a result of globalization of trade and free market practices are a major challenge for Europe [2,3]. This, together with climatic change, can increase the risk of spreading and establishing new pathogens in currently disease-free countries [4].

European and Mediterranean Plant Protection Organization (EPPO) has recorded 35 fungi in the List A1 of pests recommended for regulation as quarantine pest (absent from the EPPO region) and 32 in the List A2 (present in the EPPO region but not widely distributed). *Fusarium circinatum* Nirenberg & O'Donnell (teleomorph = *Gibberella circinata*), which causes pine pitch canker (PPC), is included in the List A2 [5]. *Fusarium circinatum* was first detected in 1945 in the south-eastern United States [6], and since then has spread widely to Haiti [7], South Africa [8], Mexico [9], Chile [10], South Korea [11], Japan [12], Uruguay [13], Colombia [14], and Brazil [15], among other regions. Spain was the first European country where the disease was detected [16,17]. The pathogen is currently established in forests in Spain, mainly in the commercial *Pinus radiata* D. Don and, to a lesser extent, in *Pinus pinaster* Ait. plantations. The pathogen has also been reported in France [18], Italy [19], and Portugal [20], although in France and Italy it is now considered to have been eradicated.

*Fusarium circinatum*, the causal agent of PPC, is now considered among the most important pathogens of Pinaceae in the world, devastating *Pinus* seedlings and mature trees in many countries globally. In fact, the European Food Safety Authority (EFSA) has established that under the current host distribution and climatic conditions, the potentially at risk areas cover over 10 million hectares of pine forests in Europe [21]. Furthermore, several studies have already highlighted that climate change will lead to increased suitability of currently disease-free areas for *F. circinatum* in the near to medium term future [22,23].

At least 60 species of *Pinus* along with *Pseudotsuga menziesii* (Mirb.) Franco are known to be susceptible to PCC [16,21,24,25]. Variation in susceptibility occurs not only among species [26–30], but also among provenances [31–35]. Thereby, taking into account the high genetic variation among the European Scots pine [36–38], Norway spruce [39], and European larch populations [40,41], testing the susceptibility of European provenances (e.g., from the Czech Republic) should be a priority.

It is well known that the occurrence of symptoms also depends on the host, as well as biotic and abiotic conditions [25]. Furthermore, the presence of asymptomatic yet infected seedlings is not unusual [42–47], which implies a high risk of PPC spread from nurseries to the field due to the latent phase of the infection. Thereby, finding those species or provenances that, not showing symptoms, might trigger the spread of the pathogen into currently disease-free countries in Europe is also a priority.

The work reported here has a twofold objective: (1) to test the susceptibility of *P. sylvestris*, *P. abies*, and *L. decidua* of Czech provenances; and (2) to check whether the presence of asymptomatic yet infected seedlings is common among these species.

## 2. Material and Methods

### 2.1. Fungal Isolates and Plant Material

The *Fusarium circinatum* isolate (FcCa6) used in this work belongs to mating type 2 (MAT-2) and was isolated from an infected *Pinus radiata* tree located in Comillas (Cantabria, Northern Spain; GPS: 4°17′17.706″ W; 43°20′5.033″ N; 265 m above sea level) [28,48–50]. Plant material consisted of seedlings of three Czech conifers: *P. sylvestris*, *P. abies*, and *L. decidua*. Scots pine seedlings were 1.5-year-old plants from South Bohemian Basins, 3.5-year-old Norway spruce seedlings from Brdy Highland (Middle Bohemia), and 1.5-year-old European larch from Bohemian-Moravian Highlands. The seedlings were obtained from a mix of seeds of cones from forest stands designated by the Czech forest legislation (Act No. 149/2003 Coll. and Decree No. 29/2004 Coll.) as "selected" (i.e., forest stand of at least 1 ha, with more than 40 trees of at least 60 years, having the phenotypic classification "A" or "B", with at least 100 m distance from any forest stand with the worst classification "D") and phenotypic classification "B". These forest stands were located at middle altitudes (500–600 m above sea level), with mean annual temperature 6.5–7.5 °C, and annual precipitations 690–800 mm [51].

*2.2. Pathogenicity Tests*

The spore suspension of *F. circinatum* was cultured on potato dextrose broth (PDB medium). For that, an Erlenmeyer flask, containing 1 L of PDB and five mycelial agar plugs (diameter 4–5 mm) obtained from the margin of an actively growing colony, was placed on an orbital shaker at 180 cycles per minute for 24 h at 25 °C. Finally, the spore suspension was obtained by filtering twice through sterile cheesecloth to remove hyphae. The spore suspension was adjusted with a hemocytometer at $10^6$ spores $ml^{-1}$.

Pathogenicity tests were carried out by the stem inoculation technique [52]. Thus, a wound was made with a sterile scalpel 5–7 cm above the collar of each plant after removal of needles from that area. Then 25 seedlings of each species in full growth were inoculated with 100 µL of the spore suspension ($10^6$ spore $mL^{-1}$) and another 25 control seedlings were mock-inoculated in the same way with sterilized distilled water instead of spore suspensions. The inoculated wound was immediately sealed with Parafilm® to prevent drying, and the seedlings were placed in a growth chamber at 21.5 °C with a 16/8 h light/dark photoperiod, following a completely randomized design. Watering and other procedures were conducted as per routine nursery practice, except that no fertilizers or fungicides were applied.

Symptoms were estimated three times per week according to the scale (slightly modified) published by Correll et al. [53] for over three months (102 days), where 0 = healthy plant, 1 = resin and/or necrosis at the point of inoculation and healthy foliage, 2 = resin and/or necrosis beyond the point of inoculation, 3 = accentuated wilting and appreciable dieback, 4 = dead plant.

The presence of asymptomatic yet infected seedlings was checked at three stages: 2, 5, and 8.5 months after inoculation. Five seedlings per species were checked at 2 months after inoculation and ten seedlings per species at 5 and 8.5 months after inoculation (except for *P. sylvestris*, because all inoculated seedlings had died until 8.5 months after inoculation). Reisolation of the pathogen was carried out from stem tissues >2.5 cm beyond the point of inoculation (upward and downward). For the reisolation, the fragments were sterilized by: dipping in sterile distilled water for 3 min, followed by shaking in 3% sodium hypochlorite (*v*/*v*) for 2 min, thereafter shaking in 70% ethanol (*v*/*v*) for 2 min, and finally dipping for 5 min in sterile distilled water to remove any remaining traces of disinfectants. The samples were then dried for 1–5 min in a sterile laminar flow cabinet on sterile filter paper, before being cut in small pieces for plating on potato dextrose agar (PDA) with 0.5 g/L of streptomycin sulfate (to prevent bacterial growth) [54]. The plates were placed in growth chambers in the dark at 25 °C and once the colonies started growing, they were subcultured on Spezieller Nährstoffarmer Agar (SNA) media, in which it is feasible to identify the distinguishing features of *F. circinatum* using a stereomicroscope (e.g., the coiled sterile hyphae) [55].

*2.3. Statistical Analyses*

Analyses of variance (ANOVAs) and multiple comparison procedures were performed to test the effects of *F. circinatum* on inoculated seedlings. The scale of symptoms was previously transformed to a quantitative variable using the area under the disease progress curve (AUDPC), calculated as the sum of the area of the corresponding trapezoids for the trial duration [56,57]. As the data violated two of the ANOVA assumptions (normality and homogeneity of variances), robust statistical methods were applied [58]. In particular, heteroscedastic one-way ANOVAs were performed using the generalized Welch procedure and a 0.1 trimmed mean transformation. The ANOVAs were carried out using the "Wilcox' Robust Statistics (WRS)" package. Survival analysis based on the nonparametric estimator Kaplan–Meier [59] was performed with the "Survival" package [60] to test the mortality up to the end of the experiment (102 days). Survival curves were created with the "Survfit" function and the differences between the curves were tested with the "Survdiff" function. All analyses were performed using R software environment (R Foundation for Statistical Computing, Vienna, Austria).

## 3. Results

*Pinus sylvestris* seedlings began to die 37 days after inoculation (days after inoculation), whereas no mortality was recorded in the control seedlings. The survival analyses revealed significant differences between treatments ($\chi^2$ = 17.5, $p < 0.001$) (Figure 1). Nearly 50 percent of *P. sylvestris* inoculated seedlings died within three months following the inoculation and no survival was recorded eight months after inoculation. Mortality was not recorded in inoculated *P. abies* and *L. decidua* seedlings during the trial. Control seedlings did not die during the experimental assay.

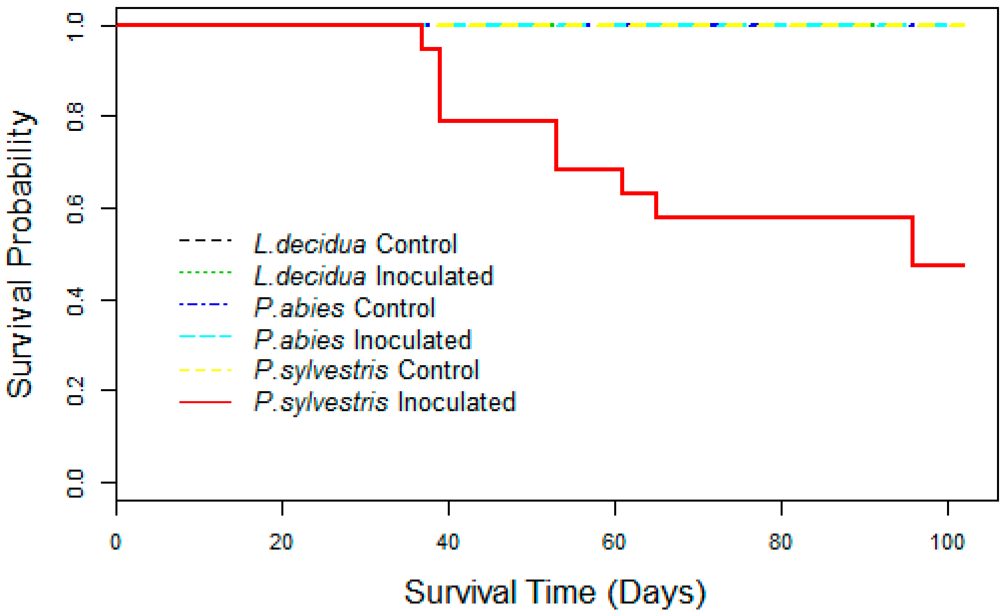


**Figure 1.** Plot of survival probability determined using the Kaplan-Meier estimate of the survival function for *Pinus sylvestris*, *Picea abies*, and *Larix decidua* seedlings infected with *Fusarium circinatum*. Note: no mortality was recorded for inoculated seedlings of *P. abies* and *L. decidua* and all control seedlings. For this reason, all of these curves overlap in a straight line making it difficult to distinguish them.

Symptomatology varied according to the treatment (Figure 2). The ANOVA revealed significant differences in the values of AUDPC caused by the *F. circinatum* inoculations. In fact, a significant interaction between species (*P. sylvestris*, *P. abies*, and *L. decidua*) and treatment (inoculated vs. control) was observed ($F$ = 29.08, $p < 0.001$) (Figure 3). Inoculated *P. sylvestris* seedlings showed the highest AUDPC values, confirming its susceptibility to *F. circinatum*. The values of AUDPC also increased in *Larix decidua* as a result of the *F. circinatum* inoculation (Figure 3). This difference seems to be only due to the presence of a higher number of seedlings showing resinosis at the point of inoculation in the inoculated seedlings than the controls (Figure 2). Inoculated *P. abies* seedlings showed resinosis even beyond the inoculation point (Figure 2), but no significant differences were found in the AUDPC values (Figure 3).

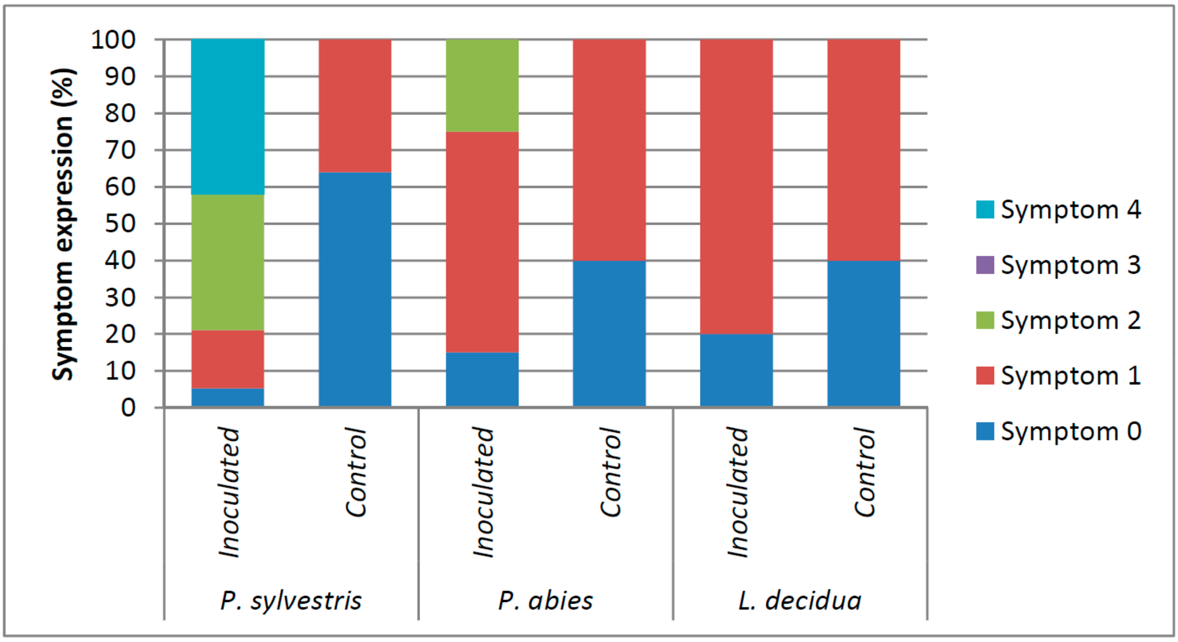


**Figure 2.** Type of symptomatology showed by *Pinus sylvestris*, *Picea abies*, and *Larix decidua* seedlings at the end of the experiment following the Correll et al. [53] scale, where 0 = healthy plant, 1 = resin and/or necrosis at the point of inoculation and healthy foliage, 2 = resin and/or necrosis beyond the point of inoculation, 3 = accentuated wilting and appreciable dieback, 4 = dead plant.

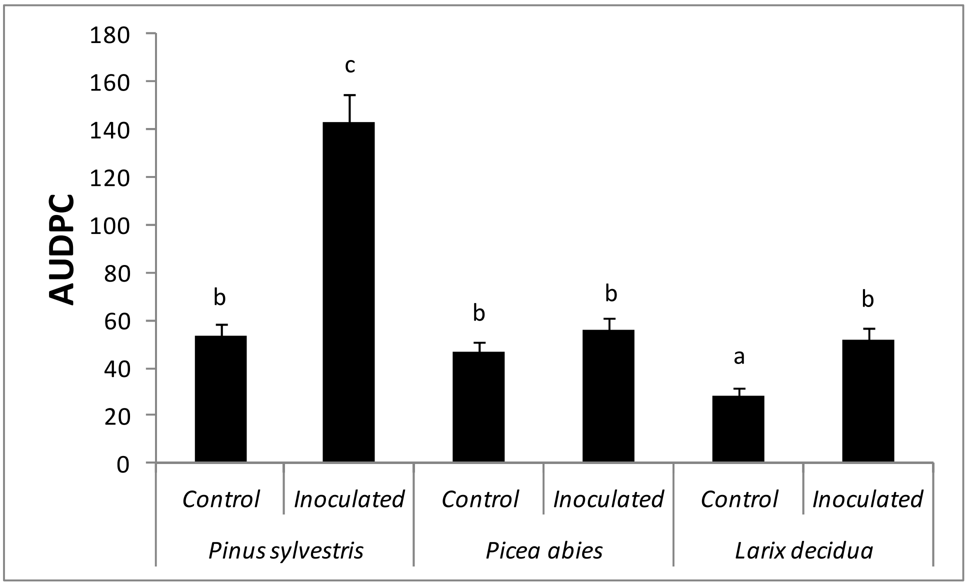


**Figure 3.** Comparison of area under the disease progress curve (AUDPC) for *Pinus sylvestris*, *Picea abies*, and *Larix decidua* seedlings. Different letters above bars indicate significantly different means (Generalized Welch procedure 0.1 trimmed means, $p < 0.05$).

Although the ratio of reisolation of *F. circinatum* declined over time, the presence of asymptomatic yet infected seedlings was confirmed in the three species up to 8.5 months after inoculation (Figure 4). *Fusarium circinatum* was reisolated beyond the inoculation point, both upward and downward.

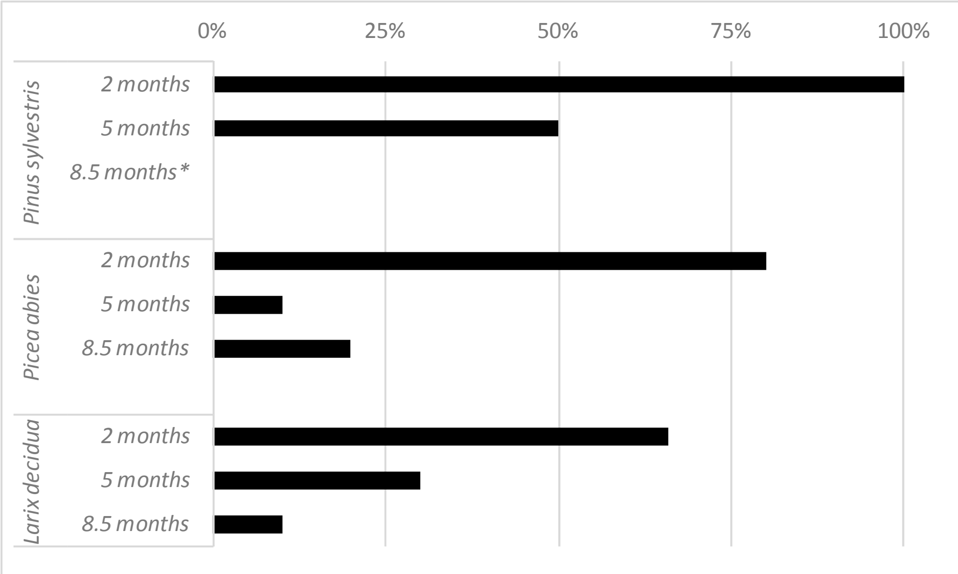


**Figure 4.** Ratio of positive reisolations of *Fusarium circinatum* from inoculated seedlings at 2, 5, and 8.5 months after inoculation. (*) All the seedlings were already dead 8.5 months after inoculation and reisolation could not be carried out.

## 4. Discussion

To our knowledge, this is the first study testing the susceptibility of conifers from the Czech Republic to *F. circinatum*. The present study demonstrated the pathogenicity of *F. circinatum* to the Czech provenance of *P. sylvestris*, which is in concordance with previous studies carried out with Spanish provenances [28–30,61]. Inoculated seedlings started dying 37 days after inoculation, although approximately half of the seedlings survived up to 5 months after inoculation. Timing of mortality as a result of *F. circinatum* inoculations varies according to the host. In fact, the mortality of *P. radiata* seedlings, the most susceptible species to *F. circinatum* [25], is commonly 7 days after inoculation [62]. However, the study findings are in contrast to the results obtained by Pérez-Sierra et al. [61] and Mullett et al. [63], who found that all or the majority (>98%) of inoculated *P. sylvestris* seedlings died 56 and 40 days after inoculation, respectively. This discrepancy could be due to the age of the seedlings. The present study used 1.5-year-old seedlings, whereas the seedlings used by Pérez-Sierra et al. [61] and Mullett et al. [63] were 7–8-month-old and one-year-old seedlings, respectively. So, this seems to point out that *P. sylvestris* might acquire age-related resistance, which had already been noted by Martín-García et al. [50].

Nevertheless, taking into account that intraspecific genotypic variation has been recognized as a source of resistance to *F. circinatum* in other pine species [33,35,45,64–66], the genetic effect should not be dismissed. In fact, Martín-García et al. [50] found that Romanian provenance of *P. sylvestris* was not susceptible to *F. circinatum* and this resistance was related to the high genetic variability of European Scots pine populations [67,68].

In forests, *F. circinatum* has been traditionally restricted to *Pinus* species and *P. menziesii* [21,25,69]. Nevertheless, damping off caused by *F. circinatum* has been also reported in other conifers. Post-emergence damping-off, but not pre-emergence, has been reported in *P. abies* [28,50]. On the other hand, Martínez-Álvarez et al. [28], in a field trial, did not find differences in the AUDPC values for 3-year-old *P. abies* seedlings between inoculated and control plots, which is consistent with the present study. The discrepancy between experiments testing the susceptibility of *P. abies* might be also due to age-related resistances. In fact, Martínez-Álvarez et al. [28] already pointed out that the differences between the results obtained from laboratory and field trials (including other conifers, not only *P. abies*) may be a result of the age of the seedlings. The resinosis beyond the point of inoculation found in ca.

25% of the inoculated seedlings confirms that *F. circinatum* is able to infect *P. abies* seedlings. However, the fact that no mortality was recorded 8.5 months after inoculation seems to indicate that *P. abies* seedlings with lignified vascular tissues are able to tolerate the *F. circinatum* infection.

The AUDPC values for *L. decidua* were significantly different between inoculated and control seedlings (Figure 3) because resinosis at the point of inoculation was more frequent in inoculated seedlings than controls (Figure 2). However, resinosis beyond the inoculation point or wilting was not observed. This, together with the fact that pre-emergence and post-emergence damping off in *L. decidua* has been demonstrated [28], seems to also indicate age-related resistances in this conifer.

The reisolation of *F. circinatum* beyond the inoculation point demonstrated that this quarantine pathogen is able to infect the three conifers tested (*P. sylvestris*, *P. abies*, and *L. decidua*), giving rise to asymptomatic yet infected seedlings for at least eight and a half months. This finding is consistent with results obtained by Elvira-Recuenco et al. [46] and Swett et al. [47] who found symptomless *P. radiata* seedlings for 475 days after inoculation or at least 52 weeks, respectively. Other studies also demonstrated the presence of asymptomatic seedlings, but all of them were conducted on *Pinus* species [42–44]. To our knowledge, this is the first study in which the presence of asymptomatic yet infected seedlings has been recorded in the *Picea* and *Larix* genera.

## 5. Conclusions

The present study demonstrated the susceptibility of the Czech provenance of *P. sylvestris* to *F. circinatum*, which confirms the importance of testing the susceptibility of European conifers both at species and provenance level. Further studies are needed to confirm the susceptibility of mature trees of *P. sylvestris* in the Czech Republic. However, the fact that *P. sylvestris* covers ca. 16% of the total forest area in the Czech Republic and that almost 80% of its regeneration is artificial (i.e., by seedlings produced in nurseries) [1] underscores the importance of carrying out surveys to monitor the eventual presence of *F. circinatum* in nurseries. Europe, and in particular the Czech authorities, should be aware of the high threat posed by this quarantine pathogen not only for the potential serious losses in forests, but also for the forest nurseries sector and the management of public greenery.

This study has also confirmed, for the first time, the presence of asymptomatic yet infected seedlings of *P. abies* and *L. decidua*. In 2007, the European Commission adopted emergency measures to prevent the introduction into and the spread within the Community of *F. circinatum* (Commission Decision 2007/433/EC of 18 June 2007). However, the measures provided within this decision are applied only for plants of the genus *Pinus* and the species *Pseudotsuga menziesii*. Therefore, the European legislation fails to control the movement of symptomless seedlings of other genera, such as *Picea* and *Larix*. This, together with the fact that these seedlings could remain symptomless for at least eight and a half months, drastically increases the risk of new introductions into disease-free European countries.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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