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Susceptibility of Several Northeastern Conifers to *Fusarium circinatum* and Strategies for Biocontrol

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Received: 11 July 2017; Accepted: 18 August 2017; Published: 30 August 2017

Abstract: *Fusarium circinatum*, the causal of pine pitch canker disease (PPC), is now considered among the most important pathogens of Pinaceae in the world. Although in Europe PPC is only established in the Iberian Peninsula, the potential endangered areas cover over 10 million hectares under the current host distribution and climatic conditions. It is therefore a priority to test the susceptibility of those species and their provenances, within Central and Northern Europe and find biological control agents (BCAs) against the disease. In this study, the susceptibility of *Pinus sylvestris*, *P. mugo* and *Picea abies* Romanian provenances to *F. circinatum* was tested using three inoculum doses. In parallel, the potential use of *Trichoderma atroviride* and *Trichoderma viride* as BCAs against *F. circinatum* was also tested. This study has demonstrated, for the first time, the susceptibility of *P. mugo* to *F. circinatum*. Likewise, the susceptibility of *P. abies* was also confirmed. The fact that the Romanian provenance of *P. sylvestris* has not been susceptible to *F. circinatum* suggests genetic resistance as a potential tool to manage the disease. This, together with the apparent effectiveness of *Trichoderma* species as BCAs, seems to indicate that an integrated management of the disease might be feasible.

Keywords: pitch canker; *Trichoderma*; provenance; pathogenicity; biological control agent; resistance

1. Introduction

Fusarium circinatum Nirenberg & O'Donnell (teleomorph = *Gibberella circinata*), which causes pine pitch canker disease (PPC), is now considered among the most important pathogens of Pinaceae in the world, devastating *Pinus* seedlings and mature trees in many countries globally [1,2]. Since the first report of pitch canker in the southeastern United States [3], *F. circinatum* has spread widely and has been reported in Haiti [4], South Africa [5], Mexico [6], Chile [7], Korea [8], Japan [9], Uruguay [10], Colombia [11] and Brazil [12], among other regions. In Europe, the first report was in Spain [13,14], where the pathogen is currently established in the forest, mainly in the commercial *P. radiata* and, to a lesser extent, in *P. pinaster* plantations. The pathogen has also been reported in France [15], Italy [16] and Portugal [17], although in France and Italy it is now considered eradicated. In Europe, *F. circinatum* is currently included in the A2 list (present in the EPPO region but not widely distributed) of pests recommended for regulation as quarantine pathogens.

The host range of *F. circinatum* is very wide, including up to 60 species of *Pinus* along with *Pseudotsuga menziesii* [1,2,18,19]. PPC is limited by the climate conditions, and particularly by cold stress [1]. In fact, PPC is not problematic in those parts of North America with colder winters even though the pathogen is presumably present [20]. The potential endangered areas cover over 10 million hectares in Europe under the current host distribution and climatic conditions [1]. Nevertheless, the potential distribution of pitch canker is expected to shift north as a result of climate change, which may include current *F. circinatum*-free areas [21].

Furthermore, other European conifers of the family Pinaceae whose susceptibility to PPC is still unknown could be affected by *F. circinatum* once the pathogen moves into their natural range. This, together with the seed-borne characteristic of this pathogen, amplifies the risk of spreading to disease-free areas via international trade of seeds and seedlings, resulting in the possibility of severe crop and yield losses in both nurseries and forests [2]. Thereby, testing the susceptibility of those species, and their provenances, with a Central and Northern distribution in Europe should be a priority. Indeed, the seriousness of the damage in the countries affected by the disease, as well as the threat posed to the disease-free European countries, has led to the granting of a COST (European Cooperation in Science and Technology) Action (FP1406 PINESTRENGTH) on this subject (www.pinestrength.eu).

Pinus sylvestris is present in nearly all European countries, covering over 28 million hectares in Europe, being one of the most commercially important species and delivering valuable ecosystem services [22]. The susceptibility of several Spanish provenances of *P. sylvestris* to *F. circinatum* has been already demonstrated [23–25]. Nevertheless, taking into account the high genetic variation among the European Scot pine populations [26–28], it would be expected that susceptibility of provenances from Central and Northern Europe might vary. *Picea abies* is the main species in the Boreal and subalpine conifer forests, from Central (in mountains) to Northern and Eastern Europe up to the Ural Mountains [29]. Like *P. sylvestris*, *P. abies* exhibits a relatively large amount of genetic variability [30] and, in particular, the Romanian population differs markedly from the other European populations [31]. Although *Pinus mugo* has a scarcer natural range than *P. sylvestris* and *P. abies*, the ecological importance of this species is well known [32]. Several root rot pathogens, such as *Heterobasidion annosum* and *Armillaria* spp., have been identified as a potential threat to *P. mugo* [33]. Nevertheless, no information about its susceptibility to *F. circinatum* is available to date.

In plant pathology, the biological control involves the use of microbial antagonists or host-specific pathogens to suppress diseases or control weed populations [34]. Biological control agents (BCAs) have been recognized as an alternative to the utilization of chemicals, which is highly restricted by European Union regulations (e.g., Directive 2009/128/EC). Amongst the BCAs, the use of *Trichoderma* spp. has been widely tested as a more environmentally friendly method to manage diseases caused by species of *Fusarium*, *Pythium*, *Sclerotinia*, *Rhizoctonia*, *Gaeumannomyces*, among others [35–37] and, in particular, *F. circinatum* [38–42]. However, the results about their efficacy have been contradictory and have varied among species.

The work reported here has a twofold objective: (1) to test the susceptibility of *P. sylvestris*, *P. mugo* and *P. abies* of Romanian provenances; and (2) to test the potential use of *Trichoderma atroviride* and *T. viride* as BCAs against *F. circinatum* on the three conifers studied.

2. Materials and Methods

2.1. Fungal Isolates and Plant Material

The *Fusarium circinatum* isolate (FcCa6) used in this work was isolated from an infected *Pinus radiata* tree located in Comillas (Cantabria, North Spain; 395568, 4798793 UTM ETRS89 spindle 30, 265 m above sea level) [25,39,43]. Two biological control agents were used in this study, in particular, *Trichoderma atroviride* (HP136; GenBank Accession number KT323338) and *T. viride* (HP155; GenBank

Accession number KT323356) [42]. Both species were isolated from a *Pinus radiata* stand in Cantabria (Spain) in 2010 (latitude N 43°20′16.2″, longitude W 4°18′17.1″). Plant material consisted of seeds from three Romanian conifers: *Picea abies*, *Pinus mugo* and *Pinus sylvestris*. The seeds used in our experiments were collected from a large number of trees from natural reserves (ca. 20–40 ha) included in the Romanian catalogue of forest genetic resources, except for those of *Pinus mugo*, which were collected from a natural stand. Further details of the provenances selected are shown in Table 1.

Table 1. Origin of the plant material used in this study.

Species	Provenance	Coordinates		Altitude	Climatic Data	
		North	West	MASL	Precipitation (mm)	Mean Temperature (°C)
Pinus sylvestris	PI-A210-2	47°40′00″	25°20′00″	1100–1400	840	5.6
Pinus mugo	Muntele Oslea	45°13′00″	22°53′00″	1850–1900	1310	3.8
Picea abies	MO C-210-2	45°15′00″	24°45′00″	1350–1650	800	4.3

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 liter of PDB and 5 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 for 24 h at 25 °C. Finally, the spore suspension was obtained by filtering twice through sterile cheesecloth to remove hyphae. Likewise, the spore suspension of *Trichoderma* species was obtained by rinsing the Petri dishes with sterile distilled water and filtering the resulting suspension [39]. A haemocytometer was used to determine the concentration of the spores in both cases (*F. circinatum* and *Trichoderma* species).

The tree seeds were first immersed in water for 24 continuous hours, replacing the water every 12 h. They were then maintained in hydrogen peroxide 3% (H_2O_2) for 15 min, washed three times with sterile distilled water and finally immersed in sterile distilled water for 30 min to remove the remaining hydrogen peroxide.

Seeds of each conifer were sown individually in germination trays (96 mL) containing a twice-autoclaved (105 kPa, 120 °C, 30 min) mixture of peat and vermiculite (1:1, *v*/*v*). The experimental design consisted of twelve treatments based on a factorial scheme with two factors at several levels: (*i*) *F. circinatum* (spore suspension): 50 spores mL^{-1}, 1000 spores mL^{-1} and 1 million spores mL^{-1} and (*ii*) Biological control agent: *Trichoderma atroviride* and *T. viride* (only one spore suspension rate, 1 million spores mL^{-1}, was used). So, twelve treatments per species (*P. abies*, *P. mugo* and *P. sylvestris*) carried out were thus: Control, *T. atroviride* (TA), *T. viride* (TV), Fc–50, Fc–10^3, Fc–10^6, Fc–50 + TA, Fc–10^3 + TA, Fc–10^6 + TA, Fc–50 + TV, Fc–10^3 + TV, and Fc–10^6 + TV. Nineteen seeds (replicates) were prepared per treatment (228 seeds per conifer, i.e., a total of 684 seeds).

Trichoderma species were added to the substrate when the seeds were sown, whereas *F. circinatum* was inoculated 15 days after sowing. In both cases, for *F. circinatum* and *Trichoderma* species, 100 μL of conidial suspension was applied on each tray cell without direct contact with the seeds.

Germination trays were incubated in a growth chamber at 21.5 °C with a 16/8 h light/dark photoperiod. They were watered three times a week, with equal amounts of tap water, throughout the study period. Germination of seeds and subsequent mortality of seedlings was recorded daily.

2.3. Statistical Analyses

Chi-square tests (χ^2) were carried out to test whether pre-emergence mortality differed among treatments. Yates' correction for continuity was applied in those cases in which the expected frequencies were below 5. Survival analysis based on the nonparametric estimator Kaplan–Meier [44] was performed with the "Survival" package [45] to test the post-emergence mortality up to the end of the experiment (38 days). Survival curves were created with the "Survfit" function and the differences between the curves were tested with the "Survdiff" function. Effect of *Trichoderma* species was only analyzed in those pine species whose susceptibility to *F. circinatum* was demonstrated in the previous step. All analyses were performed using R software environment (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

Inoculations with *F. circinatum* did not cause significant changes in pre-emergence mortality in any conifer species at any inoculum dose (data not shown). However, the application of the BCAs improved the emergence rate in some treatments of *P. abies* and *P. mugo*, although not in *P. sylvestris*. In particular, *P. mugo* showed a marked improvement in emergence for one BCA treatment: "Fc–10^3 + TV" vs. "Fc–10^3" (95% vs. 42%, respectively; χ^2 = 12.18, p < 0.001), as did *P. abies* for a number of BCA treatments: "Fc–10^3 + TA" vs. "Fc–10^3" (89% vs. 58%, respectively; χ^2 = 4.89, p = 0.03), "Fc–10^3 + TV" vs. "Fc–10^3" (100% vs. 58%, respectively; χ^2 = 7.75, p < 0.01) and "Fc–10^6 + TA" vs. "Fc–10^6" (89% vs. 53%, respectively; χ^2 = 6.27, p = 0.01).

Survival analyses demonstrated the susceptibility of *P. abies* and *P. mugo* to *F. circinatum* even in the lowest inoculum doses (50 spores mL^{-1}) (χ^2 = 7.6; p < 0.01 and χ^2 = 4.9; p < 0.03) (Figure 1a,b). However, the Romanian provenance of *P. sylvestris* was not susceptible to *F. circinatum* at any dose (χ^2 = 3.4; p = 0.34) (Figure 1c).

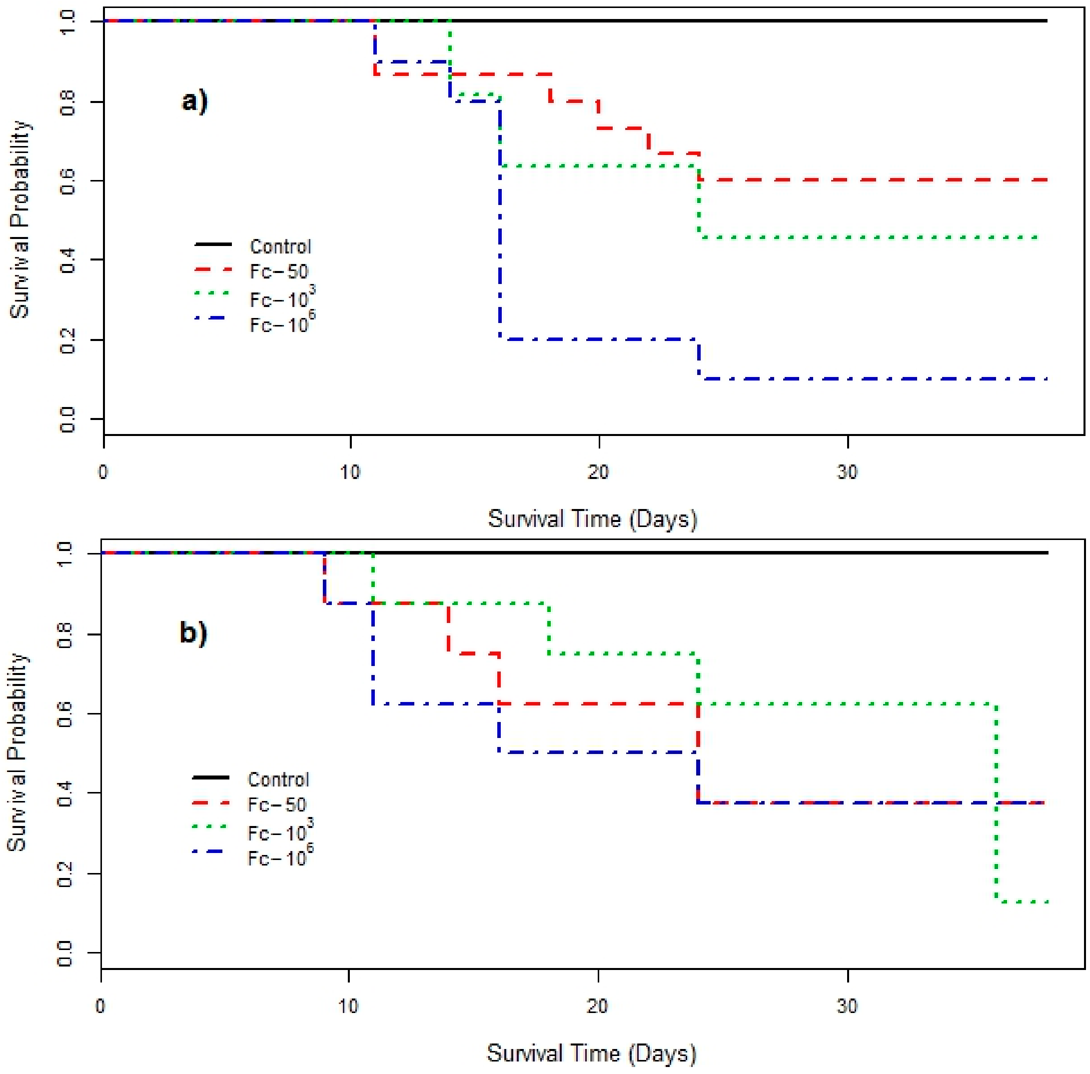

Figure 1. *Cont.*

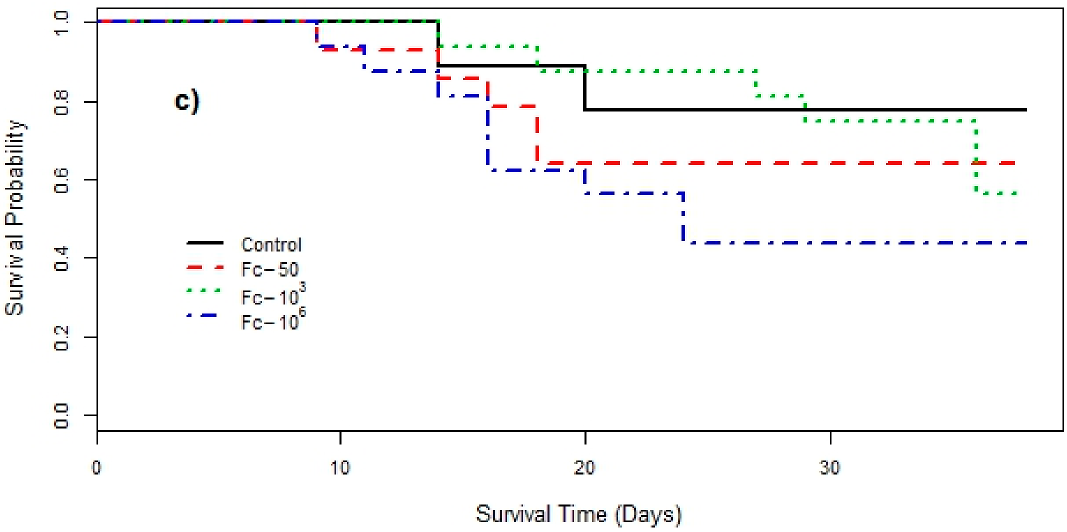

Figure 1. Plot of survival probability determined using the Kaplan-Meier estimate of the survival function for (**a**) *Picea abies*; (**b**) *Pinus mugo* and (**c**) *Pinus sylvestris* seedlings infected with *Fusarium circinatum* in relation to the inoculum dose (50, 1000 and 1 million spores mL^{-1}).

The application of *Trichoderma* species reduced the post-emergence mortality in the two susceptible species (*P. abies* and *P. mugo*). However, different results were obtained according to the inoculum dose of the pathogen and *Trichoderma* species used. In *P. abies* in the treatment at a lowest inoculum dose (50 spores mL^{-1}), neither of the two *Trichoderma* species had a significant effect on the seedling mortality (χ^2 = 3.8; p = 0.15) (Figure 2a). However, at the intermediate inoculum dose (1000 spores mL^{-1}), both *T. atroviride* and *T. viride* showed an antagonistic effect on the pathogen, reducing the post-emergence mortality (χ^2 = 5.4; p = 0.02 and χ^2 = 5.0; p = 0.03, respectively) (Figure 2b). A similar pattern was also found at the highest inoculum dose (1 million spores mL^{-1}), since the mortality seedlings decreased as a result of the application of both *Trichoderma* species (χ^2 = 14.3; $p < 0.001$ and χ^2 = 8.3; $p < 0.01$, respectively) (Figure 2c).

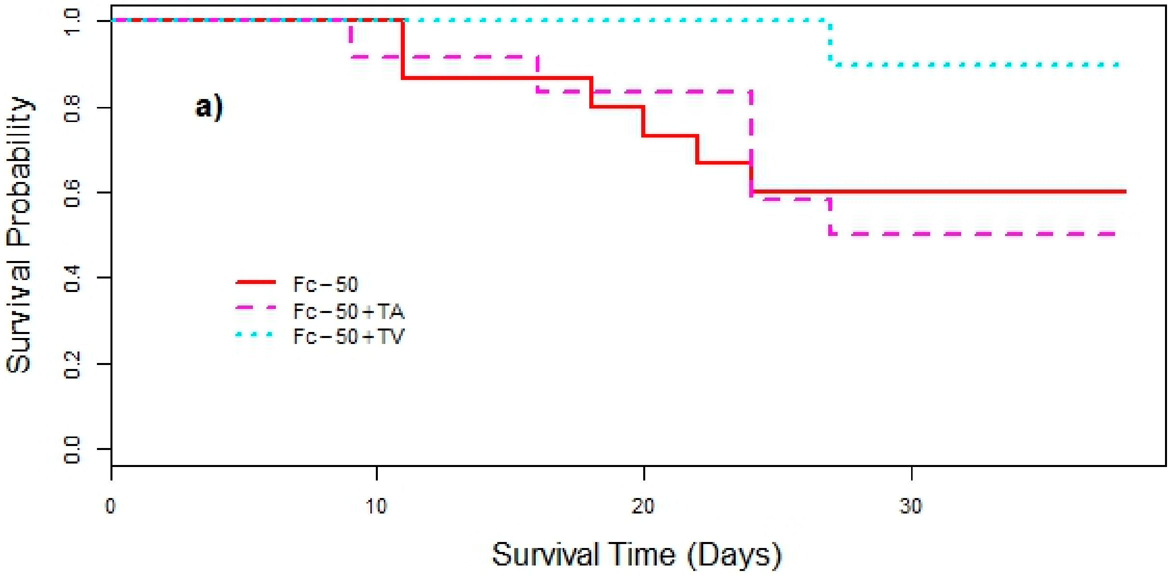

Figure 2. *Cont.*

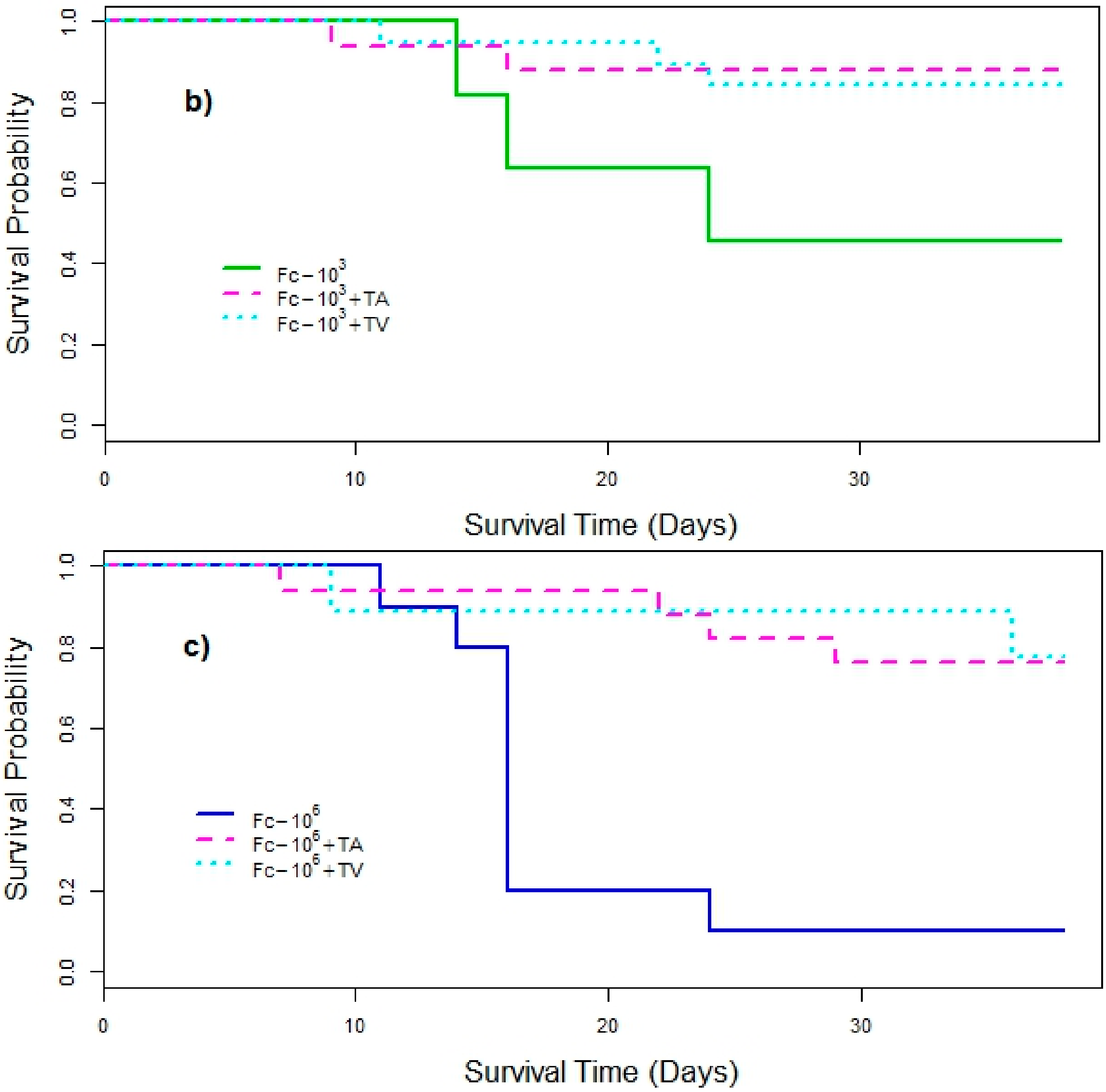

Figure 2. Plot of survival probability determined using the Kaplan-Meier estimate of the survival function for *Picea abies* seedlings infected with *Fusarium circinatum*, in presence/absence of *Trichoderma atroviride* and *Trichoderma viride*, according to the inoculum dose; (**a**) 50 spores mL^{-1}; (**b**) 1000 spores mL^{-1} and (**c**) 1 million spores mL^{-1}.

On the contrary, in the *P. mugo* treatment at the lowest inoculum dose (50 spores mL^{-1}), only *T. viride* decreased the seedling mortality, inhibiting entirely the *F. circinatum* effect (χ^2 = 7.6; $p < 0.01$). However, *T. atroviride* did not have a significant effect on the post-emergence mortality to this inoculum dose (χ^2 = 1.3; p = 0.26) (Figure 3a). Nevertheless, the opposite pattern was found at the intermediate inoculum dose (1000 spores mL^{-1}). Whereas *T. atroviride* caused lower mortality rates (χ^2 = 9.7; $p < 0.01$), no significant effect was found when *T. viride* was applied (χ^2 = 1.8; $p < 0.17$) (Figure 3b). Regarding the highest inoculum dose tested (1 million spores mL^{-1}), neither of the two *Trichoderma* species had a significant effect on the seedling mortality (χ^2 = 1.1; p = 0.58).

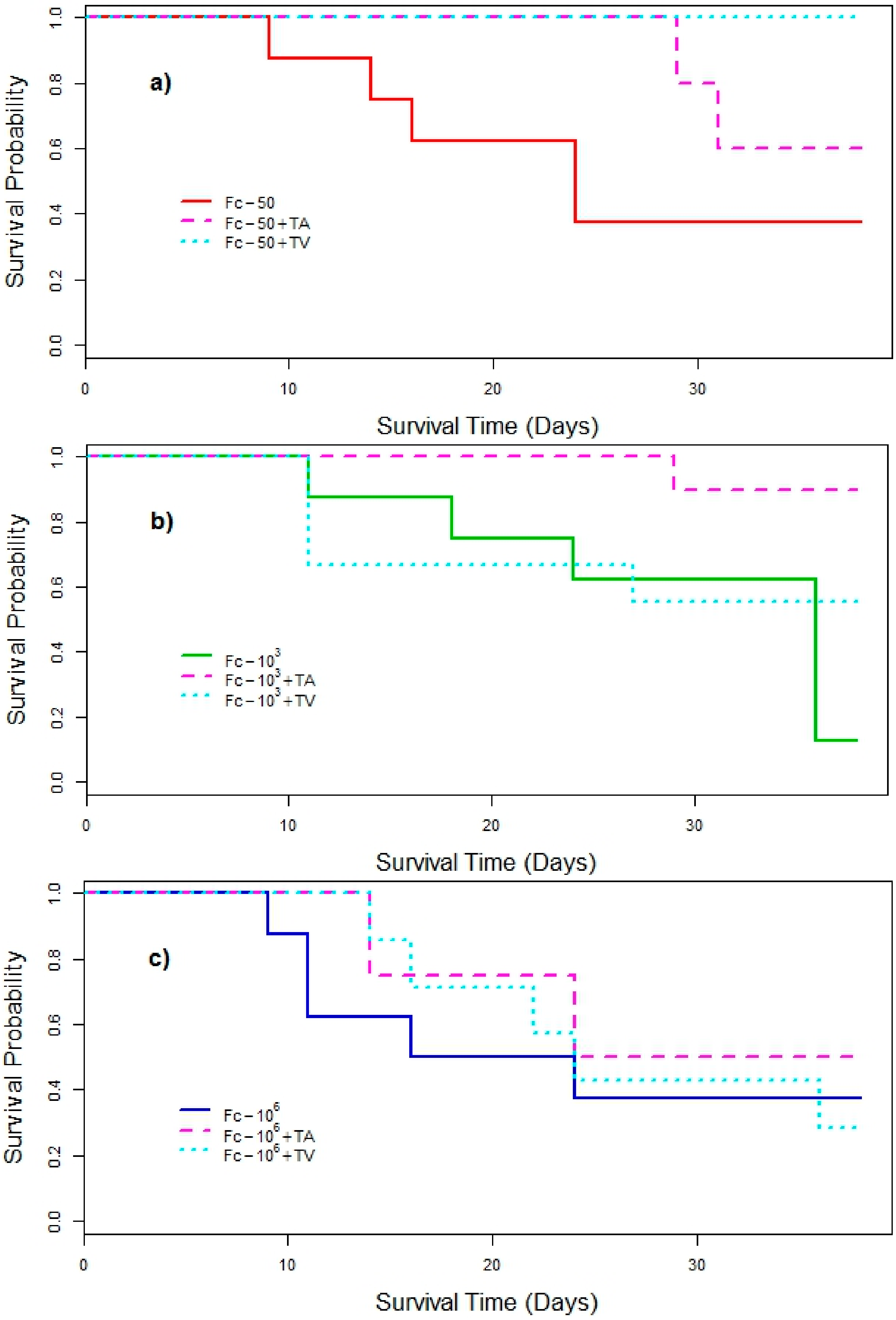

Figure 3. Plot of survival probability determined using the Kaplan-Meier estimate of the survival function for *Pinus mugo* seedlings infected with *Fusarium circinatum*, in presence/absence of *Trichoderma atroviride* and *Trichoderma viride*, according to the inoculum dose; (**a**) 50 spores mL^{-1}; (**b**) 1000 spores mL^{-1} and (**c**) 1 million spores mL^{-1}.

4. Discussion

Invasive alien species are estimated to have cost the EU at least €12 billion per year over the past 20 years [46]. Hence, special attention must be paid to invasive forest pathogens, which have increased exponentially in the last four decades as a result of globalization of trade, climate change and free market policies [47–49]. To date, the current distribution of *F. circinatum* is restricted to the Iberian Peninsula. However, as noted above, the potential distribution of pitch canker could shift north under climate change predictions, reaching Central and Northern Europe [21]. Furthermore, climatic suitability is only a limiting factor for its establishment in the field. However, under artificial conditions inside greenhouses, forest nurseries are threatened by *F. circinatum* across Europe. Therefore, to know the susceptibility of species and provenances from Central and Northern European countries, such as Romania, is essential.

To our knowledge, this study is the first pathogenicity test performed with *F. circinatum* and *P. mugo* seedlings, and the results demonstrate the susceptibility of mugo pine to PPC. Although this species has a smaller distribution range than other pines, its ecological importance is beyond any doubt. In fact, its extensive root system with many branches consolidating loose soils plays a great role in preventing torrents and avalanche erosions on high mountains [32].

The host range for *F. circinatum* has been traditionally restricted to *Pinus* species and *Pseudotsuga menziesii* [1,2,18]. Nevertheless, the present study has also demonstrated that *Picea abies* seedlings are susceptible to *F. circinatum*, at least at an early age. This pattern was also found by Martínez-Álvarez et al. [25] in an essay with the provenance "East Europe". Moreover, they did not find any effect of *F. circinatum* on seedling emergence either. This outcome takes on greater relevance taking into account that *P. abies*, together with *P. sylvestris*, is the conifer more produced by the forest nurseries and planted in Central and Northern Europe [50,51].

On the other hand, the Romanian provenance of *P. sylvestris* was not susceptible to *F. circinatum*, having neither pre-emergence nor post-emergence mortality. These results differed from those of Martínez-Álvarez et al. [25], who found that the emergence rate of a Spanish provenance was lower when the substrate was inoculated with *F. circinatum* than in the controls. These authors also found post-emergence mortality after 90 days. Bearing in mind that the *F. circinatum* isolate was the same in both studies, this discrepancy could be due to a genetic effect, since Spanish provenances differ genetically from northern and eastern European provenances [52,53]. The effect of genetic variability on the susceptibility to *F. circinatum* has been previously demonstrated in other pine species [54–59]. This finding highlights the importance of testing the susceptibility not only at species level, but also at provenance level across Europe.

Iturritxa et al. [24,60] demonstrated that two-year-old seedlings of *P. sylvestris* were relatively susceptible to *F. circinatum*. This fact, together with the Martínez-Álvarez et al. [25] research, who found that damages in one-year-old inoculated and control seedlings were not different, seems to point out that *P. sylvestris* might acquire age-related resistance, in which a maturing plant or plant organ becomes less susceptible to pathogens [61].

Several biocontrol mechanisms are used by *Trichoderma* spp. to effect disease control: mycoparasitism, antibiosis, promoting plant growth, competition for nutrients and space, and plant defense responses [35–37,62]. Hence, *Trichoderma* has been used as a BCA against a broad range of pathogens, both fungi (*Fusarium* sp., *Rhizoctonia* sp., *Botritis* sp., *Sclerotinia* sp., etc.) and oomycetes (*Phytopththora* sp., *Phytium* sp., etc.) [34–37,62,63].

Several *Trichoderma* species, including *T. atroviride* and *T. viride*, have been already tested as BCAs against *F. circinatum* in *P. radiata* [39–42,64]. However, to our knowledge this is the first study that performs and demonstrates the antagonistic effect of *Trichoderma* species on *F. circinatum* in other hosts. In fact, the present study found that both *T. atroviride* and *T. viride* reduced the post-emergence mortality in *P. abies* and *P. mugo*. However, their effectiveness varied according to the pine species and inoculum dose of the pathogen. In *P. abies*, the antagonistic effect of *Trichoderma* species was only observed at the highest inoculum doses (1000 and 1 million spores mL^{-1}). This could be due to the fact that at the lowest inoculum dose (50 spores mL^{-1}), the post-emergence mortality rate was relatively low (40%; in comparison to the other two treatments ca. 55 and 90%, respectively) to detect a significant antagonistic effect caused by the *Trichoderma* species. However, *P. mugo* showed an opposite pattern, with the *Trichoderma* species being more efficient at the lowest inoculum doses.

The results of this study are in contrast to the results obtained by Martínez-Álvarez et al. [39], who did not find any antagonism exerted by *T. viride* in *P. radiata* seedlings. Taking into account that in both studies the same *F. circinatum* isolate was used, this was probably due to the fact that they inoculated simultaneously the pathogen and *T. viride*. The importance of timing in the application of the BCA was already reported by Moraga-Suazo et al. [40]. While *Trichoderma* strains exerted a reduction of the post-emergence mortality when they were added to substrate 7 days before adding the pathogen, no significant effect was found when they were added 48 h after the pathogen. Likewise, Iturritxa et al. [64] also pointed out the importance of growing the *Trichoderma* species during one week before inoculating the pathogen in an in vitro assay. These facts demonstrate the potential of *Trichoderma* species as a preventive formulation to avoid the PPC appearance in forest nurseries, suggesting that the timing of its application is important.

The disparity in the results could also be due to the different environmental conditions and/or the duration of the experiments. While López-López et al. [41] found that the presence of *T. asperellum* leads to a reduction in disease incidence on recently emerged seedlings of *P. radiata* (similar conditions to our study), Mitchell et al. [38] reported a sharp decrease in the antagonistic effect of a *T. harzianum* application in the field after 180 days. In a similar vein, Martínez-Álvarez et al. [42] found that promising *Trichoderma* strains used in in vivo experiments did not exert any antagonism on *F. circinatum* in field, which could be due to the seedling age (two-year-old *P. radiata* seedlings) or the environmental conditions.

5. Conclusions

The present study confirms the importance of testing the susceptibility of European conifers whose susceptibility to *F. circinatum* is still unknown. In fact, here has been demonstrated, for the first time, the susceptibility of *P. mugo* to *F. circinatum*. Likewise, the susceptibility of *P. abies* was also confirmed. Furthermore, although more research is needed to confirm that the Romanian provenance of *P. sylvestris* has resistance to *F. circinatum*, this seems to point to genetic resistance as a potential tool to manage the disease. This, together with the evidences of the effectiveness of *Trichoderma* species as BCAs against the disease, seems to indicate that an integrated management of the disease might be feasible. Notwithstanding the promising findings, further studies are needed to (*i*) confirm the susceptibility of mature trees of *P. mugo* and *P. abies*, (*ii*) elucidate the role of the genetic factors on the resistance found in the Romanian provenance of *P. sylvestris*, and (*iii*) confirm the effectiveness of *T. atroviride* and *T. viride* against *F. circinatum* in a wide range of environmental conditions and their persistence in the long term.

Acknowledgments: This article is based upon work carried out during COST Action FP1406 PINESTRENGTH (Pine pitch canker-strategies for management of *Gibberella circinata* in greenhouses and forests), supported by COST (European Cooperation in Science and Technology). This study was funded by The Ministry of Economy and Competitiveness of the Government of Spain (MINECO) under projects AGL2012-39912 and AGL2015-69370-R and also by the INTERREG-SUDOE PLURIFOR project. The Portuguese Foundation for Science and Technology (FCT) supported Jorge Martín-García (Post doc grant-SFRH/BPD/122928/2016). Marius Paraschiv was awarded with a Short Term Scientific Mission within the PINESTRENGTH framework to perform the experiment in the Laboratory of Forest Pathology at the University of Valladolid (Spain).

Author Contributions: Jorge Martín-García, Marius Paraschiv, Juan Asdrúbal Flores-Pacheco, Danut Chira, Julio Javier Diez and Mercedes Fernández conceived and designed the experiment. Jorge Martín-García, Marius Paraschiv, Juan Asdrúbal Flores-Pacheco, Julio Javier Diez and Mercedes Fernández performed the experiments. Jorge Martín-García analyzed the data and wrote the paper. All authors reviewed the paper.

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

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