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Aggressiveness of *Fusarium oxysporum* and *F. verticillioides* isolates on stone and scots pine under greenhouse conditions

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Abstract

Scots pine (*Pinus sylvestris*) and Stone pine (*Pinus pinea*) are two important species used in re-forestation that are subject to damage by damping-off fungi in forest nurseries. Twenty-two isolates of *Fusarium oxysporum* and *F. verticillioides* from diseased seedlings of eight different hosts were tested for aggressiveness on seeds and seedlings of both pine species, including the effects on seedling emergence and mortality. Scots pine was more susceptible to damping-off than Stone pine, as indicated both by reduced seedling emergence and elevated seedling mortality. The impact of *F. oxysporum* and *F. verticillioides* on seeds and seedlings did not differ significantly for either pine species. Our findings support previous studies that found that these are damping-off pathogens on the studied pines. Whereas most isolates proved to be pathogenic, some isolates of both *Fusarium* species showed to be non-pathogenic.

KEYWORDS
aggressiveness, emergence, *fusarium oxysporum*, *fusarium verticillioides*, mortality, pathogenicity studies, *Pinus pinea*, *Pinus sylvestris*, resistance

1 | INTRODUCTION

Scots pine (*Pinus sylvestris* L.) is widespread on the Iberian Peninsula at elevations ranging from 1000 to 2000 masl and under similar climatic conditions elsewhere in northern Europe (Montero & Martínez, 2000). On the other hand, Stone pine (*Pinus pinea* L.) is one of the most common species at lower altitude in coastal and continental areas of central Spain and is highly valued for its edible seeds (Abad Viñas et al., 2016). Recent decades have seen increasing demands for seedlings of both pine species for use in re-forestation of former agricultural lands in many rural areas of Europe. This process is supported by the Green Deal Program of the European Union (European Commission, 2021) as an alternative to the traditional agricultural systems, to promote diversification of production and to further economic development of the rural area.

Reliable production of pine seedlings requires nursery practices that minimize the impact of diseases, which includes careful attention to sanitation. Among the disease problems of greatest concern is damping-off, which significantly contributes to yield losses by causing vascular wilt in forest nurseries throughout the world (Nef & Perrin, 1999; Stewart et al., 2012; Tahat et al., 2021). Species of *Fusarium* are well-known as causal agents of damping-off (Dick & Dobbie, 2002; Machón et al., 2009), which can manifest itself as reduced rates of seedling emergence and/or post-emergence mortality, along with needle blight and root rot (Gordon et al., 2015; Luo & Yu, 2020). These pathogens may cause important losses in nurseries, and latent infections may later become symptomatic in plantations (Landeras et al., 2005). Their mechanism of action is based upon the production of a secondary metabolite known as fusaric acid (FA), a phytotoxin that can display strong toxicity in plants (López-Díaz et al., 2018). During the infection process, the fungus also secretes various specific compounds, such as cell-wall-degrading enzymes and mycotoxins, that potentially play important roles in its pathogenicity (Zuriegat et al., 2021).

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Fusarium oxysporum (Fo) Schlecht. Emend. Snyd. & Hans has been ranked among the top 10 fungal pathogens in molecular plant pathology based on scientific/economic importance, causing vascular wilt disease in more than 100 different crops (Dean et al., 2012; Husaini et al., 2018). Its ample host range varies from vegetables, ornamentals, fruit and field crops (Haapalainen et al., 2016; Melo et al., 2019; Rana et al., 2017; Wang et al., 2008; Yadav et al., 2019; Zhang et al., 2021). But, despite showing a broad host range, strains of Fo are highly host-specific and are genetically and morphologically different (Kistler, 1997), a distinctive characteristic of the fungus that allow to cluster individual strains with the same host range into groups called formae speciales (Edel-Hermann & Lecomte, 2019). On the other hand, *Fusarium verticillioides* (Sacc) Niremberg (=*F. moniliforme* Sheldon) is well known for causing infections that reduce germination percentage (Venturini et al., 2013) and post emergence damping-off and root rot in maize (El-Demerdash et al., 2017). Particularly, it produces fumonisin mycotoxins, a secondary metabolite that is even implicated in human diseases (Blacutt et al., 2018).

The presence of *F. oxysporum* and *F. verticillioides* in European forest nurseries has previously been reported (Martín-Pinto et al., 2006; Nef & Perrin, 1999). *Fusarium oxysporum* has generally been considered the most damaging species in this genus, although *F. verticillioides* can also be an important cause of disease of forest seedlings in nurseries (Martín-Pinto et al., 2008; Pawuk, 1978). The effect of these two species on Scots, and especially on Stone pine emergence and survival of seedlings has not been well studied.

Intraspecific variation in aggressiveness in *Fusarium* spp. has been frequently observed, with some strains being non-pathogenic saprophytes (Edel-Hermann et al., 1997, 2001). Consequently, to understand the role species play in causing disease one must start with confirmation of pathogenicity. Here, we present results of tests designed to evaluate the aggressiveness of *Fusarium oxysporum* and *F. verticillioides* isolates on Stone and Scots pine under greenhouse conditions.

2 | MATERIALS AND METHODS

2.1 | Fungal strains and host plants

In total, 12 isolates of *F. verticillioides* and 10 isolates of *F. oxysporum* were obtained from diseased seedlings from one of eight different green emergence (when green tissue of seedling was observed), and mortality were recorded weekly hosts growing in forest nurseries in northern Spain (Table 1). Single conidium sub-cultures of each isolate were maintained on potato dextrose agar (PDA) at 23°C in

TABLE 1 Code name, host and location of the *Fusarium* isolates used in the aggressiveness test on Scots and Stone pine.

Isolate	Species	Host	Origin	Climate[a]	
				T (°C)	HR (%)
Fo-1	*F. oxysporum*	*Pinus nigra*	*Valladolid*	7.5	75.0
Fo-2	*F. oxysporum*	*P. nigra*	*Soria*	6.2	72.3
Fo-3	*F. oxysporum*	*P. nigra*	*Valladolid*	7.5	75.0
Fo-4	*F. oxysporum*	*P. nigra*	*Soria*	6.2	72.3
Fo-5	*F. oxysporum*	*P. nigra*	*Valladolid*	7.5	75.0
Fo-6	*F. oxysporum*	*P. pinea*	*Soria*	6.2	72.3
Fo-2P	*F. oxysporum*	*Quercus ilex*	*Soria*	6.2	72.3
Fo-4P	*F. oxysporum*	*Q. pyrenaica*	*Soria*	6.2	72.3
Fo-5P	*F. oxysporum*	*P. pinea*	*León*	6.3	76.5
Fo-6P	*F. oxysporum*	*P. nigra*	*Valladolid*	7.5	75.0
Fv-1	*F. verticillioides*	*P. nigra*	*Valladolid*	7.5	75.0
Fv-3	*F. verticillioides*	*P. pinea*	*Valladolid*	7.5	75.0
Fv-4	*F. verticillioides*	*P. pinea*	*Soria*	6.2	72.3
Fv-5	*F. verticillioides*	*P. pinaster*	*Soria*	6.2	72.3
Fv-6	*F. verticillioides*	*P. pinea*	*Valladolid*	7.5	75.0
Fv-7	*F. verticillioides*	*P. radiata*	*Valladolid*	7.5	75.0
Fv-8	*F. verticillioides*	*P. uncinata*	*Valladolid*	7.5	75.0
Fv-2P	*F. verticillioides*	*P. nigra*	*Valladolid*	7.5	75.0
Fv-3P	*F. verticillioides*	*P. sylvetris*	*Valladolid*	7.5	75.0
Fv-4P	*F. verticillioides*	*P. nigra*	*Soria*	6.2	72.3
Fv-5P	*F. verticillioides*	*P. pinea*	*Soria*	6.2	72.3
Fv-6P	*F. verticillioides*	*P. pinea*	*León*	6.3	76.5

[a]HR, relative humidity; T, temperature. Both are daily means during the sampling periods.

a growth chamber as described by Chala et al. (2011), before starting the experiment. Pine seeds were obtained from a commercial forest nursery (Viveros Fuenteamarga S.L.) in Cabezón de Pisuerga, Valladolid, Spain. The seed provenances ES.08 (Montaña Soriano-Burgalesa) for *P. sylvestris* and ES.01 (Meseta Norte) for *P. pinea* were selected for the experiment.

2.2 | Aggressiveness test

To produce inoculum, monosporic isolates were subcultured on PDA in 9 cm Petri plates and incubated at 23°C in the dark. After 7 days, 15 agar plugs from each colony margin were placed into an Erlenmeyer flask containing 200 mL of potato dextrose broth (PDB) and cultured at 23°C in the dark in an orbital shaker at 200 rpm to stimulate sporulation (Muñoz-Adalia et al., 2018). Five days later, microconidia of each isolate were collected by filtration through glass fibre (90 µm), and their density was estimated microscopically using a haemocytometer and adjusted through the addition of sterile distilled water (SDW) to obtain a suspension with 10^6 microconidia mL^{-1}.

Seeds of Scots and Stone pine were surface sterilized with 30% H_2O_2 for 30 and 60 min, respectively, due to the lower hardiness of Scots seeds, and then washed five times with SDW. Afterwards, seeds were sown in 200 mL containers (AGRIPROTECTOR S.L.) filled with peat moss. Five millilitres of the suspension of either *F. oxysporum* or *F. verticillioides* were deposited directly in the container next to the pine seeds. Control pots were treated with 5 mL of SDW. After inoculation, no pesticide was applied. The experiment was installed under a completely randomized design with factorial arrangement of the treatments (*Fusarium* strains vs. pine species). Each treatment was represented by four replicates of 10 seeds per container of either Stone pine or Scots pine, inoculated with *F. oxysporum* or *F. verticillioides*. All containers were randomly arranged on a bench and maintained in a greenhouse for 11 weeks seedling for the duration of the experiment. At the end, the pathogen was re-isolated only from inoculated seedlings. As a whole, an isolate was regarded as aggressive if it induced significantly lower emergence or greater mortality than the water controls.

2.3 | Data analysis

Seedling emergence and mortality data sequences, as response variables, were studied by a repeated measures analysis using the software Statistica 7.0 (99 ed., StatSoft Inc.) to test for variation over time of the effect of *Fusarium* isolates on both pine species. At the end of the trial (11 weeks), one-way analysis of variance (ANOVA) and Dunnett test ($p \leq .05$) was run using the XLStats software to compare the effect of each isolate against the control treatment.

3 | RESULTS

3.1 | Seedling emergence

In general, emergence began the second week after seeds were sown for both pine species and continued until the seventh week in Scots pine and the ninth week in Stone pine, with significant influence of time on emergence ($p \leq .05$), where Scots pine presented the fastest germination and emergence.

Cumulative emergence at the end of the trial (11th week) showed that Scots pine seedlings exhibited a higher susceptibility to *Fusarium*, as revealed by the high number of *Fusarium* isolates (21 out of the 22 tested; $p \leq .05$), which caused a significant reduction on emergence relative to controls (Figure 1). Isolate Fo-4P caused the lowest Scots pine emergence (12.5%; $p = .000$), followed by isolate Fv-8 (16.25%; $p = .000$) and Fo-2P (18.75%; $p = .000$), whereas isolate Fo-3 (61.25%; $p = .999$) did not significantly affect the emergence. In Stone pine, only 16 *Fusarium* isolates showed lower emergence than the control, with isolates Fv-6 (42.50%; $p = .00$), Fv-7 (45.63%; $p = .00$) and Fv-3P (46.25%; $p = .00$) the most aggressive to this species (Figure 2).

3.2 | Seedling mortality

Cumulative seedling mortality was first observed in the 5th and 6th week after sowing for Scots and Stone pine, respectively, with significant effect of time on the number of dead plantlets in both pine species ($p \leq .05$). At the final time point (11 weeks), most *Fusarium*

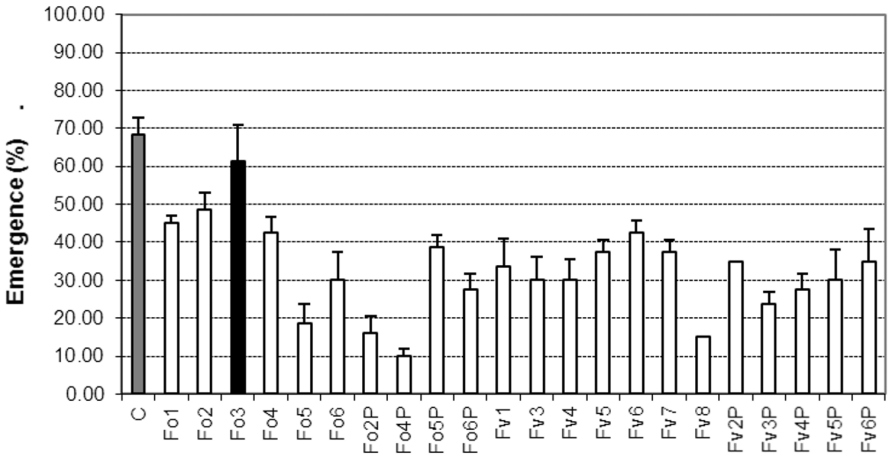


FIGURE 1 Emergence (%) in Scots pine (*P. sylvestris*) 11 weeks after inoculation with different *Fusarium* isolates. Black bars are not significantly different from controls according to Dunnett test ($p > .05$).

isolates (18 out of 22, $p \leq .05$) had shown a significant effect on mortality of Scots pine. Here, isolate Fo5 induced the highest rate of mortality (59.21%, $p = .00$), followed by Fo4P (58.09%, $p = .00$) and Fo2P (55.77%, $p = .00$). Only the treatments with Fo-1 (2.78%, $p = .081$), Fo-2 (0.00%, $p = .354$), Fo-3 (0.00%, $p = .999$) and Fo-4 (3.12%, $p = .052$) failed to show differences relative to the control in both the pine species (Figures 3 and 4). In Stone pine, overall mortality was lower than in Scots pine, and fewer isolates (10 out of 22, $p \leq .05$) were aggressive to seedlings of this species.

Scots pine was clearly more susceptible to *Fusarium* damage than Stone pine based on differences in the number of seedling emergence and post-emergence mortality. In general, isolates that caused damage on Stone pine also affected Scots pine, the exception being isolate Fo-4 which caused significant mortality only on Stone pine, showing the interaction effect ($p \leq .05$) between fungal damage and pine species (Table 2).

4 | DISCUSSION

Both Scots and Stone pine were susceptible to damage caused by *Fusarium* spp., with Scots pine being more severely affected, both

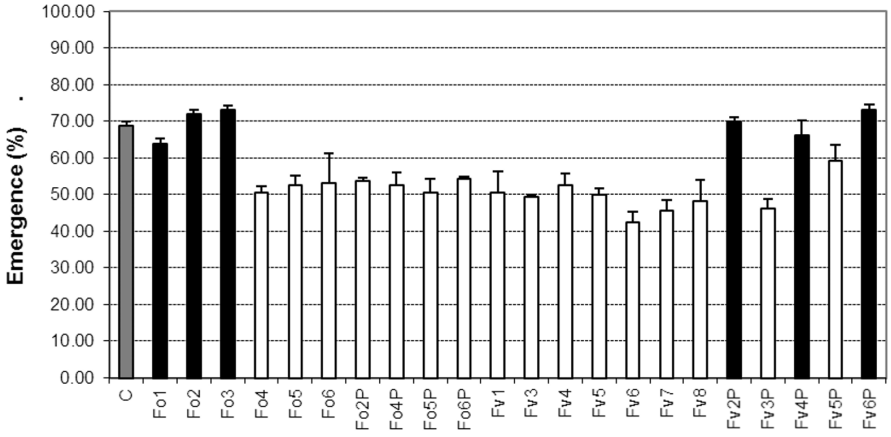


FIGURE 2 Emergence (%) in Stone pine (*P. pinea*) 11 weeks after inoculation, with different *Fusarium* isolates. Black bars are not significantly different from controls according to Dunnett test ($p > .05$).

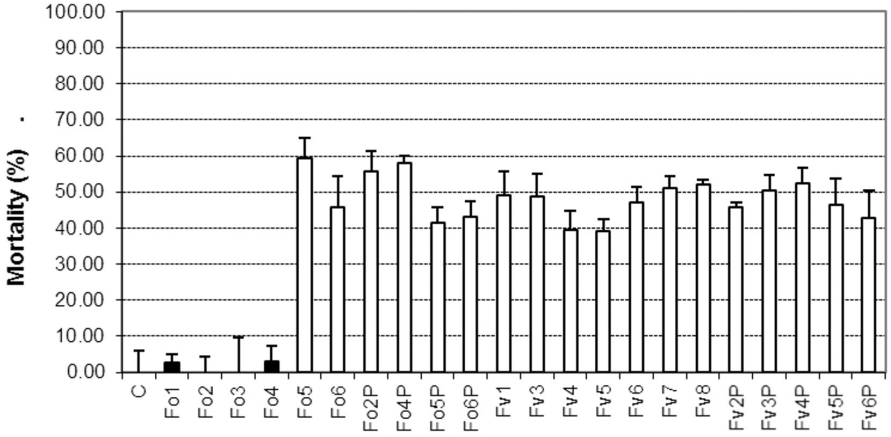


FIGURE 3 Mortality (%) in Scots pine (*P. sylvestris*) seedlings 11 weeks after inoculation with different *Fusarium* isolates. Black bars are not significantly different from controls according to Dunnett test ($p > .05$).

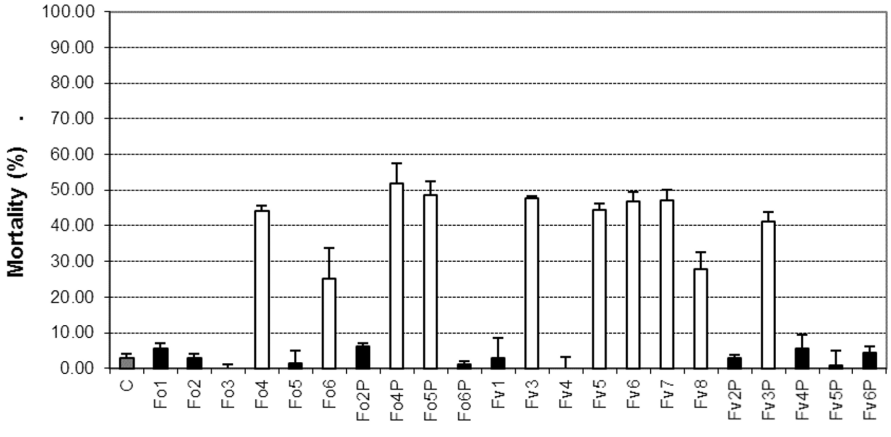


FIGURE 4 Mortality (%) in Stone pine (*P. pinea*) seedlings 11 weeks after inoculation with different *Fusarium* isolates. Black bars are not significantly different from controls according to Dunnett test ($p > .05$).

TABLE 2 Mean squares and probability for the main effect and interactions of *Fusarium* strains and pine species on the emergence and mortality of the plant seedlings.

Variable	Source	Df	Mean square (MS)	Probability (p)
Emergence	*Fusarium* strain (F)	21	6105.4	.000
	Pine species (P)	1	10363.7	.000
	F × P	21	136.5	.000
	Error	132	35.6	
Mortality	*Fusarium* strain (F)	21	4.5389	.000
	Pine species (P)	1	37.9593	.000
	F × P	21	2.8789	.000
	Error	132	.6309	

Note: Data converted through arcsine transformation.
Abbreviation: df, degrees of freedom.

in terms of pre- and post-emergence damping-off. These results are in agreement with previous studies (Kacprzak et al., 2001; Manka et al., 2001). Likewise, Magnani (1975) had observed that *P. pinea* was more resistant to damping-off by *F. oxysporum* than *P. pinaster*, *P. brutia*, *P. halepensis*, *P. canariensis*, *P. radiata* and *P. wallichiana*. Despite this higher resistance of *P. pinea* to damping-off, in our assays, emergence and post-emergence survival were severely affected in both species. This points out the need for effective management of these fungi either by biological control (Carvalho et al., 2014; Khillare et al., 2021), chemical control (Gote et al., 2021; Swiecimska et al., 2020) or abiotic stress approaches, which refer to the plant defence triggered by plant-derived substances, previously synthesized in response to some abiotic stress, that induce the secretion of fungal catabolic enzymes and cause a rapid stopping of the pathogen metabolism (Stepien & Lalak-Kańczugowska, 2021; Ray et al., 2022).

The pathogenicity of *F. oxysporum* may depend on the production of enzymes, such as the tomatinase Tom1, that is involved in the infection process of the host and is required for full aggressiveness in tomato (Pareja-Jaime et al., 2008). In the case of *F. verticillioides* on maize or soybean, or *F. circinatum* on pines, the pathogenicity increases with the amount of inoculum present in seeds (inoculum potential), which negatively affects the emergence and survival of the crops (Pedrozo & Little, 2017; Woodward et al., 2022).

Emergence of Scots pine seeds was affected significantly by all *Fusarium* isolates except isolate Fo-3. The germination/emergence stage was revealed as the most sensitive for this pine since older stages were less affected. The higher susceptibility of Scots pine during germination may be related to the relatively small size of its seeds (5.7–7.2 g per 1000 seeds; Farinha et al., 2018) as compared to those of Stone pine (867.0 ± 8.0 g per 1000 seeds; Udval & Batkhuu, 2013). Thus, the larger Stone pine seeds with greater reserves may deploy more effective defensive measures, and the thicker testa may limit the rate of fungal penetration.

Inconsistent pathogenic behaviour according to the host growth stage has also been found in other studies. For example, in tissues infected by *F. verticillioides*, only the infections occurring at seedling and young plant stages of maize showed high radicle decay ability (Venturini et al., 2013), although Sousa et al. (2021) recently found that in the reproductive phenological stages, the plant was more susceptible to the infection of the same pathogen. Infections of *F. oxysporum* at the early seedling growth stage of *Lupinus luteus* showed that the disease was particularly aggressive after the change from autotrophic to heterotrophic phase of the plant, that is, up to 72 h of growth (Morkunas et al., 2004). Likewise, when employing the ectomycorrhiza *Laccaria laccata* as a biocontrol against *F. oxysporum* in *Pinus pinea* at various growth stages, there was a successful achievement at 18 weeks after sowing, although the treatment failed to control the disease at pre-emergence and late damping-off stage (Machón et al., 2009).

Emergence of Scots pine was faster than that of Stone pine, with the first seedlings appearing in all the treatments at the 3rd and 4th week, respectively. Also, the first symptoms of mortality were observed earlier in Scots pine (5th week) than in Stone pine (6th week). These results may be related to the natural faster emergence of Scots pine but may also show the higher specific susceptibility of this species. It is also known that many plants have evolved a protection system to combat fungal infections and possess intricate mechanisms which, once triggered by the pathogen, transduce signals to activate a defence pathway that involves specific proteins (Husaini et al., 2018).

Both *Fusarium* species were pathogenic on Scots and Stone pine, and the aggressiveness of *F. verticillioides* isolates was not different from that of *F. oxysporum*. The absence of significant pathogenic differences between these two species has also been shown for *Plantago psyllium* (Elwakil & Ghoneem, 1999), *P. nigra* (Martín-Pinto et al., 2004) and *P. palustris* (Pawuk, 1978). Therefore, although *F. oxysporum* has been traditionally regarded as the most pathogenic species of the genus, *F. verticillioides* should be regarded as a potential contributor to a damping-off complex, in the absence of evidence to the contrary.

Although all *Fusarium* isolates used in this study were obtained from diseased seedlings, some proved to be only weakly or non-pathogenic, as in the case of isolate Fo-3. Many isolates obtained from diseased seedlings of other pine species (*P. nigra*, *P. pinaster*, *P. uncinata and P. radiata*) did not significantly damage seeds or seedlings of Scots and Stone pine (Machón et al., 2009), whereas

isolates from *Quercus ilex* and *Q. pyrenaica* (Fo-2P and Fo-4P) were among the most damaging to Scots pine. Thus, our data indicate that the host range of some *F. oxysporum* isolates includes both pines and oaks, which argues against application of the *forma specialis* concept (Booth, 1971), that is, the ability to cause disease in a unique host. In this sense, further studies with a larger sampling of isolates and hosts would be needed to better assess the validity of this concept for species of *Fusarium* associated with seedling diseases in pines.

Our findings support previous studies that found that *F. oxysporum* and *F. verticillioides* are damping-off pathogens on Scots and Stone pines. Their pathogenicity on seeds and seedlings of the pines reveals wide differences among isolates, including some with no pathogenic effect. It also provides new information on the susceptibility of Stone pine to pre- and post-emergence damping-off caused by *Fusarium* spp. The aggressiveness of *F. verticillioides* on both pine species is reported for the first time, while Stone pine was observed to be more resistant to damping-off than Scots pine. Apparently, the aggressiveness of isolates of either species was not related to the host of origin.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Gene Bank at https://www.ncbi.nlm.nih.gov/genbank/.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/efp.12831.

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