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***K. V. DAVYDENKO*[1,2]**

PRELIMINARY ASSESSMENT OF PATHOGENICITY OF *FUSARIUM CIRCINATUM* ON GERMLINGS OF *PINUS SYLVESTRIS* IN UKRAINE

[1]*Ukrainian Research Institute of Forestry and Forest Melioration named after G. M. Vysotsky*
[2]*Kharkiv Zooveterinary Academy*

Fusarium circinatum is the causal agent of Pine Pitch Canker (PPC) of pines and established in the Iberian Peninsula in Europe only. However, it is hypothesized that its range could expand through the Europe in the near future. The disease is very harmful to forests and nurseries all around the world. Despite the aggressiveness of this fungus, no trials on the susceptibility of Ukrainian Scots pine provenances from *Pinus sylvestris* to *F. circinatum* have been performed. The first preliminary pathogenicity test was carried out in vitro and revealed the strong probability of high level of pine seedlings susceptibility to pitch canker.
Keywords: *Fusarium circinatum*, Pine Pitch Canker (PPC), Scots pine (*Pinus sylvestris*), damping-off.

Introduction. Conifers are native components of the tree flora in Europe, and Scots pine (*Pinus sylvestris*) usually forms the dominant vegetation cover and are thus important components of the ecosystems of Ukraine. Recently, the amount of new invasive species in Europe has significantly increased, which, in turn, brought about emerging of new destructive diseases in forest stands (Santini et al. 2013). Among them *Fusarium circinatum* Nirenberg & O'Donnell (sexual morph: *Gibberella circinata* Nirenberg & O'Donnell ex Britz, T.A. Cout., M.J. Wingf. & Marasas) could pose a serious threat to the ecological and economic sustainability of forest ecosystem.

The pathogen is believed to originate from North America where it was first discovered. Recently, it has been found in Central America, South America, South Africa, East Asia and Europe. This pathogen can devastate pine plantations. Its outbreak has been recently reported in Spain and Portugal (EPPO 2012). It was also detected in urban parks on *P. halepensis* and *P. pinea* in Italy (Gordon et al. 2011), and on *P. menziesii* and *Pinus* spp. in French nurseries (Gordon 2006). However, the disease has been officially eradicated in these countries. Thus, further entry into Europe, and spread within the EU are considered very likely.

Several pathways for entry have been identified i.e. the import of contaminated seed and other propagation material, different forms of wood material, plant material for decorative purposes, soil and growing substrates (Ennos 2015). Furthermore, the rapid spread via such vectors like wind, wind-blown rain, insects and other animals carrying spores are also important pathways. Climate change will certainly predispose trees to the attack of pathogens and likely help their spread into new areas (Gordon et al. 2011, Ennos 2015).

Pitch canker caused by the ascomycete fungus *F. circinatum* infects a wide range of pine species. It has also been recorded on more than 57 other pine species, including Mediterranean species like Aleppo pine (*P. halepensis*) and Maritime pine (*P. pinaster*), as well as in the hemiboreal forests of Europe Scots pine (*P. sylvestris*), including various American and Asian species planted in Europe (Enebak & Stanosz 2003, Ioos et al. 2013). *F. circinatum* has also been reported as the causal agent of a severe root disease in seedlings of *P. radiata* and *P. pinaster* planted in nursery (Landeras et al. 2005). All pines growing in Europe can be potentially affected by the disease, with *P. Radiate* the most susceptible species (Gordon et al. 2012, Iturritxa et al. 2011) and *P. canariensis* and *P. pinea* the least susceptible ones (Wingfield et al. 2008). Differences in susceptibility among provenances of the same *Pinus* species have been reported for *P. Sylvestris* (Davydenko et al. 2018, Martínez-Álvarez et al. 2017) and *P. Pinaster* (Storer et al. 1999).

Generally, up to 60 conifers have been reported to be vulnerable to the PPC (Gordon 2006). Infection can only occur when trees are injured, and their damaged tissues are exposed to spores. Such wounds can be caused by insect damage, hail or wind damage or some mechanical means

(Storer et al. 1998). Vectors and wounding agents may vary greatly between locations and can greatly influence the impact of the disease once it arrives in a new area (Gordon 2006).

The disease is only established in the Iberian Peninsula, but it is hypothesized that its range could expand through Europe in the near future. Therefore, these areas are posed at risk, which is confirmed by climatic data and host distribution (EPPO, 2012). Moreover, other European countries including Ukraine are also at the risk of infection. Taking into account the high susceptibility of young pines to the disease and the ease of getting infected via seeds and seedlings, the threat of *F. circinatum* to the European nurseries seems very serious due to the fact that infected nurseries were typically the first point of entry of PPC with its further spread to forest plantations (Martínez-Álvarez et al. 2017, Davydenko et al. 2018).

Scots pine is an ecologically and economically important species, in particular for wood production in Ukraine. That is why a widespread distribution of *F. circinatum* may cause a major threat to the biodiversity of Ukrainian forest ecosystems, and especially in nurseries where Scots pine is the most common species. It might result in serious plant and yield losses on planting susceptible species (*Pinus* spp.) in infected areas. Thus, the nurseries infected by *F. circinatum* seem to be the first step of the disease and they make a reservoir for the transmission of the PPC to the forest plantations.

The aim of our research was to broaden the knowledge about a new potential invasive disease of pine species, as well as to evaluate the potential threat of *Fusarium circinatum*, which might be harmful to seeds and seedlings of *Pinus sylvestris,* and to test the pathogenicity of *F. circinatum* using germlings of *Pinus sylvestris.*

Materials and Methods. *Fungal isolate*. Isolate of *F. circinatum (FcCa6)* has been obtained from Spain (host – *Pinus radiata*, Comillas, Cantabria, Spain, 43°20'16.2" N and 4°18'17.1" W), from Depository of Sustainable Forest Management Research Institute, University of Valladolid – INIA, Palencia, Spain. The cultivation of the fungus *F. circinatum* as an infection material for the experiment was carried out under laboratory conditions by growing the fungi on potato dextrose agar (PDA) for 7 days in Petri dishes at 24.5°C. Preliminary assessment of pathogenicity of *F. circinatum* for pine germlings was carried out in the Research Centre of Quarantine, Invasive and Genetically Modified Organisms, Institute of Plant Protection - NRI (Poznan, Poland). For the *in vitro* virulence test, we followed the methods of James et al. (1995, 2000), which are briefly described below.

In vitro virulence test. Stratified seeds of *P. Sylvestris* from Ukrainian ecological provenances (Sumy and Kharkiv provenances in Eastern Ukraine, taken from gene reserve forests of *P. sylvestris*) were placed inside a new mesh bag, soaked in tap water to remove seed contaminants. After purification, seeds were germinated on moistened, sterile Whatman No. 3 filter paper within sterile Petri dishes and incubated at about 22°C. A single germinant (primary root about 10 mm long) was carefully placed inside Petri dishes on moistened filter paper. Fifteen-day pine seedlings were transferred to Petri dishes amended with a piece of fungal inoculums 5×5 mm (Fig.1).

Fig. 1 – Preliminary pathogenicity testing of the susceptibility of Ukrainian provenances of Scots pine to *Fusarium circinatum* (Kharkiv provenance left and Sumy provenance right)

In total, 24 Petri dishes with 48 pine seedlings (two in each) were used for preliminary assessment of pathogenicity. Sterile water was added as needed and capped Petri dishes were incubated under diurnal light (12/12) at about 23°C. Production of disease symptoms (root rot, damping-off, and no disease, respectively) was monitored and evaluated daily over 28 days.

After 28 days, all remaining germlings were harvested and cultured, as well as examined for disease symptoms, and re-isolations onto PDA medium were made from all inoculated germlings.

All inoculated and non-inoculated germlings were assessed for height growth, root parameters and dry weight.

DNA Extraction, Purification and PCR Amplification. The sampling of all germlings inoculated *Fusarium circinatum* and from control were homogenized by cutting with scissors and shaking followed by adding of two glass beads to every screw cap tube. The subsamples of 500–700 uL from all of the homogenized samples were transferred into 2 ml screw cap tubes. When all groups were ready, all tubes were put into the freeze-drier overnight (using Parafilm to keep the lids in place). All samples were used for conventional PCR tests in order to detect presence/absence of *F. circinatum* using specific primers (FCIR1 and FCIR 4 described below).

Ground or drilled material (500 to 700 mg) was transferred to a sterile microfuge tube. DNA extraction and purification were done using NucleoSpin® Plant II Midi kit (MACHEREY-NAGEL product). DNA quantification and quality control of the DNA the samples were analyzed spectrophotometrically by the NanoDrop.

The concentration of genomic DNA was determined using a spectrophotometer. DNA in individual samples was diluted to 5–10ng/µl.

Water solutions DNA received were used for the implementation of conventional PCR using species-specific primers to detect presence/absence of *F. circinatum* in samples. The specific primers forward FCIR-F (TCG ATG TGT CGT CTC TGG AC) and reverse FCIR-R (CGA TCC TCA AAT CGA CCA AGA) were used to amplify IGS rDNA region (Ioos et al. 2013). The PCR reaction mixture includes 1× PCR buffer supplied with the DNA polymerase, 0.25 mM each dNTP, 2 mM MgCl2, 0.5 µM of each CIRC1A and CIRC4A primers, 0.05 U/µL DNA polymerase and 6.0 µL of template DNA. The PCR reaction was carried out in a thermocycler and included an initial denaturation at 95°C for 3 min, followed by 40 cycles for denaturation at 95°C for 30 sec, annealing at 64°C for 55 sec and elongation at 72°C for 50 sec. A final elongation step was done at 72°C for 12 min. The PCR products were separated by electrophoresis in a 1% agarose gel.

A DNA template containing *F. circinatum* DNA should yield a 146 bp fragment after a FCIR-F and FCIR-R specific primers.

*Statistical Analyses.*Analyses of variance (ANOVAs) and multiple comparison procedures were performed to test the effects of *F. circinatum* on infested germlings. As the data violated two of the ANOVA assumptions (normality and homogeneity of variances), robust statistical methods were applied. In particular, heteroscedastic one-way ANOVAs were performed using the generalized Welch procedure and a 0.1 trimmed mean transformation. Survival analysis based on the nonparametric estimator Kaplan–Meier was performed with the "Survival" package to test the probability of mortality up to the end of the experiment (182 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).

Results. The obtained results showed that the growth of pine germlings inoculated with *Fusarium circinatum* varied considerably (Table 1), but in 28 days *F. circinatum* in this assay killed all the pine germlings. During the observation period, all inoculated germlings formed a long necrotic lesions (8.4–8.7 mm) and showed typical symptoms as basal needle dieback and wilting. The final measurement of the necrotic lesion was not carried out due to the damping-off of the majority of germlings. Moreover, *F. circinatum* strongly affected plant development, and the root growth resulted in 92.5–96.7 % of mortality on the 28th day.

Table 1

Effects of tested *Fusarium circinatum* isolate on *Pinus sylvestris* germlings growth and root development

Isolate	Kharkiv provenance				Sumy provenance			
	Root parameters (inhibition, %	Mortality, %	Height. cm	Dry weight. mg	Root parameters (inhibition, %	Mortality, %	Height. cm	Dry weight. mg
Fusarium circinatum	88.7 ± 6.8[a]	92.5 ± 12.1[a]	7.9 ± 0.2 [a]	1.7 + 0.02 [a]	86.4 ± 7.8 [a]	96.7 ± 6.9 [a]	8.5 ± 1.2 [a]	1.5 ± 0.3 [a]
Control	0 [b]	0[a]	8.3 ± 0.1 [a]	1.8 ± 0.03 [a]	0 [b]	0 [b]	8.9 ± 1 [a]	1.6 ± 0.3 [a]

Note. Bars with different letters indicate significantly different means (HSD Tukey post hoc test, α = 0.05)

The HSD Tukey post hoc test showed that the mortality level of plants and their root inhibition were almost equal for Kharkiv and Sumy provenances (Table 1). No significant difference between the root parameters for the two provenances was noticed (F = 2.73, p-value = 0.24), as well as between other parametres (F = 1.92, p-value = 0.64, F = 2.35, p-value = 0.21, F = 2.45, p-value = 0.34 for mortality, height and dry weight respectively). Generally, the mortality of pine germlings was very high, which indicates a strong effect of *F. circinatum* on the root growth and extensive vascular discolouration in root tissue and seedling viability (Fig. 2).

The highest percentages of pine germlings mortality were recorded for both provenances (92.5–96.7 % Kharkiv and Sumy respectively) (Table 1). Plant growth was reduced significantly (p = 0.97) by the *F. circinatum*, where more than 95 % germlings died and the rest of them were weakened. No dead or weakened seedlings were observed in control.

The root growth was inhibited by *F. circinatum* intensively (88.7 % for Kharkiv provenance and 86.4 % for Sumy provenance) comparing with non-inoculated germlings (Table 1). Thus, there was a strong effect of *F. circinatum* on the root growth and extensive vascular discolouration in root tissue (p = 0.98). The statistical analysis showed no significant differences between pine provenances in the root growth variable (p = 0.002) and a high probability of seedlings mortality in all tested groups (Fig. 2).

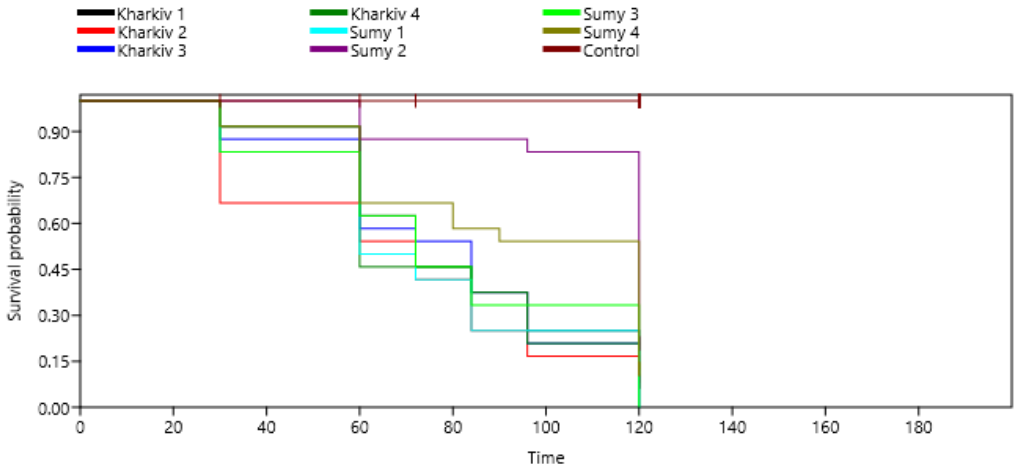

Fig. 2 – Plot of survival probability determined using the Kaplan-Meier estimate of the survival function for *Pinus sylvestris* (Kharkiv and Sumy provenances) infected with *Fusarium circinatum*

Note: no mortality was recorded for all control germlings. For this reason, all of these curves overlap in a straight line making it difficult to distinguish them

Discussion. Previous studies done in Europe, US and Asia have been reported considerable differences between susceptibility of different pine species to *F. circinatum* (Gordon et al. 1998,

Enebak & Stanosz 2003, Iturritxa et al. 2013) indicating high or moderate level of virulence and aggressiveness of *F. circinatum* for seedlings and older plants (Perez-Sierra et al. 2007, Iturritxa et al. 2013, Davydenko et al. 2018). On the other hand, some authors (Landeras et al. 2005) demonstrated that the pathogen in nurseries was isolated only from pines *P. radiata* and *P. pinaster*, while *P. nigra, P. sylvestris* and *Pseudotsuga menziesii* seedlings did not show any symptoms of pitch canker. So, it's too early to talk about statistical distribution modelling of PPC in Europe and particularly in Ukraine. Most of the distribution models use presence-only data along with environmental predictors (e.g. precipitation, temperature) across a user-defined landscape that is divided into grid cells. By using presence-only records and data about susceptible species in Europe, it is assumed that the current distribution of the pathogen indicates its ecological requirements, as different authors cite contradictory data. Our results are too preliminary and there is no enough evidence to generalize the data on its pathogenicity level. However, our study demonstrated that *F. circinatum*, if appeared in Ukraine, might be an important pathogen of *P. sylvestris* in nurseries within a short period (Fig. 2). For the most part, all studies rely on inoculation data in greenhouse or field result in a brief and clear assessment of pathogen to pose a serious threat to the plants, so further experiments are needed. For that reason, we are planning to inoculate seedlings from Ukraine to continue our study and search for a resistant genotype to pitch cancer (Swett and Gordon 2013).

Thus, our study showed that *F. circinatum* caused significantly higher mortality of 15-day old pine germlings than non-inoculated seedlings in the control group ($p < 0.05$). Therefore, based on the preliminary assessment of symptoms development, we stated that Ukrainian provenances of *P. sylvestris* might be significantly susceptible to pitch canker. The reason for this could be that plants were too young to develop proper physiological mechanism responsible for the induction of plant resistance. Furthermore, observed absence of variation in the susceptibility to pitch canker provides the prospect for greater utilization of genetic breeding for the management of this disease in the future. Moreover, a few works demonstrate that infection of *F. circinatum* can be in the latent phase. We can say that these findings are good indicators to make an effort and perform breeding programmes in Ukraine now in order to be ready in the future, when the introduction of the disease to pine forests becomes a fact.

Some studies indicate the pine shoot beetle (*Tomicus piniperda*) and other bark beetles as potential vectors of the pathogen during maturation, feeding on the shoots of healthy pine trees (Landeras et al. 2005, Romón et al. 2007, Bezos et al. 2015). Since *T. piniperda* is one of the most common bark beetles in Ukraine, it will be able to increase the potential threat of the diseases pathogen spread within the country.

Conclusions. The results of the study indicate that in future *Fusarium circinatum* might be the most important pathogen causing damping-off of Ukrainian pine seedlings, and a serious threat for the Ukrainian foresty in case of its introduction to the country.

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Давиденко К. В.[1,2]

ПОПЕРЕДНЄ ОЦІНЮВАННЯ ПАТОГЕНОСТІ ІНВАЗІЙНОГО ГРИБА *FUSARIUM CIRCINATUM* ДЛЯ ПРОРОСТКІВ *PINUS SYLVESTRIS* В УКРАЇНІ

[1]*Український науково-дослідний інститут лісового господарства та агролісомеліорації ім. Г. М. Висоцького*

[2]*Харківська Державна Зооветеринарна академія*

Fusarium circinatum є інвазійним збудником виразкового рака сосни, який був уперше знайдений в Іспанії та Португалії, але ймовірним є подальше поширення фітопатогена у хвойних насадженнях Європи найближчим часом. Патоген уражує насіння, саджанці, молодняки й стиглі насадження хвойних порід. Незважаючи на високу агресивність цього гриба, в Україні ще не проводили випробування на сприйнятливість сосни звичайної до *F. circinatum*. Метою нашої роботи було проведення попереднього оцінювання агресивності й вірулентності сіянців сосни звичайної з генетичних резерватів Харківської та Сумської областей *in vitro*. Ці рослини виявили високий рівень вилягання сходів сосни під час штучного інфікування патогеном, що свідчить про ймовірну високу сприйнятливість саджанців сосни до цього збудника.

К л ю ч о в і с л о в а : *Fusarium circinatum*, виразковий сосновий рак, сосна звичайна (*Pinus sylvestris*).

Давиденко Е. В.[1,2]

ПРЕДВАРИТЕЛЬНАЯ ОЦЕНКА ПАТОГЕНОСТИ ИНВАЗИВНОГО ГРИБА *FUSARIUM CIRCINATUM* ДЛЯ СЕЯНЦЕВ *PINUS SYLVESTRIS* В УКРАИНЕ

[1]*Украинский научно-исследовательский институт лесного хозяйства и агролесомелиорации им. Г. М. Высоцкого*

[2]*Харьковская Государственная Зооветеринарная академия*

Fusarium circinatum является инвазивным возбудителем язвенного рака сосны, который был впервые найден в Испании и Португалии. Вполне вероятно его дальнейшее распространение в хвойных насаждениях Европы в ближайшее время. Болезнь поражает семена, саженцы, молодняки и спелые насаждения хвойных пород, вызывая полегание сеянцев и саженцев и язвенный некроз более взрослых насаждений. Несмотря на высокую агрессивность этого гриба, в Украине еще не проводились испытания на восприимчивость сосны обыкновенной к *F. circinatum*. Целью нашей работы было проведение предварительной оценки агрессивности и вирулентности сеянцев сосны обыкновенной из генетических резерватов Харьковской и Сумской областей *in vitro.* Эти растния показали высокую смертность при искусственном инфицировании патогеном, что свидетельствует о вероятной высокой восприимчивости саженцев сосны к этому возбудителю.

Ключевые слова: *Fusarium circinatum*, язвенный сосновый рак, сосна обыкновенная (*Pinus sylvestris*).

E-mail: kateryna.davydenko74@gmail.com

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