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SPHAEROPSIS SHOOT BLIGHT IN PINE PLANTATIONS UNDER STRESS CONDITIONS

Davydenko K. V. Sphaeropsisshoot blight in pine plantations under stress conditions. *Sphaeropsis/Diplodiashoot blight (SSB)damages shoots and needles of different pines (Pinussp.). The aim of this study was: to classify correctly the Sphaeropsis species occurring on different pine species and regions of Ukraine; to determine whether Sphaeropsis shoot blight can pose a threat to pine plantations under stress conditions; and to detect latent infection Sphaeropsis sapinea in symptomless pine shoots. The polymerase chain reaction (PCR) protocol was used in research using specific primers.Only S. sapinea was confirmed to be a causal agent of Sphaeropsis shoot blight in pine plantation in three regions of the left-bank Ukraine. Our study confirmed also the presence of latent infection in symptomless pine shoots and the possibility of its activation and disease outbreak under environmental factors such as hail, windstorm and water stress ....……………………………………………………21 Ref.*
Keywords: Sphaeropsis / Diplodiashootblight (SSB), *Sphaeropsissapinea*, latentinfection.

Давиденко К. В. Діплодіоз у насадженнях сосни у стресових умовах. *Диплодіоз уражує пагони та хвою різних видів сосни (Pinussp.). Метою досліджень було уточнити видовий склад збудників діплодіозуу соснових культурах різних регіонів України, установити можливість збільшення ризику розвитку хвороби під впливом стресу та виявити латентну інфекцію у безсимптомних пагонах сосни. За допомогою молекулярних методів дослідження з використанням специфічних праймерів підтверджено існування у насадженнях трьох областей Лівобережної України одного виду збудників діплодіозу — Sphaeropsis sapinea, наявність латентної інфекції S. sapinea у безсимптомних пагонах сосни, а також можливість її активування під впливом посухи, пошкодження градом, вітромта іншими чинниками. ………………………………………………21назв.*
Ключові слова: діплодіоз, *Sphaeropsis sapinea*, латентна інфекція.

Давиденко К. В. Диплодиоз в насаждениях сосныв условияхстресса. *Диплодиоз поражает побеги и хвою различных видов сосны (Pinus sp.). Целью исследований было уточнить видовой состав возбудителей диплодиоза* в *сосновых культурах разных регионов Украины, установить возможность увеличения риска развития болезни под влиянием стресса и обнаружить латентную инфекцию в бессимптомных побегах сосны. С помощью молекулярных методов исследования с использованием специфических праймеров подтверждено существование в насаждениях трех областей Левобережной Украины одного вида возбудителей диплодиоза — Sphaeropsis sapinea, наличие латентной инфекции S. sapinea в безсимптомных побегах сосны, а также возможность ее активации под влиянием засухи, механического повреждения градом, ветром и другими факторами…………………………………………………………………………..21назв.*
Ключевыеслова: диплодиоз, *Sphaeropsis sapinea*, латентная инфекция.

Introduction. During last decades, some invasive fungal pathogensof conifers, such as *Dothistroma pini* Hulbary and *D. septosporum* (Dorog.) Moreleton, have reached Ukraine and other European countries [8, 21], attributable not only to climate change, but also to increasing global trade[21]. However, some other forest infectious diseases became widely distributed in Ukraine, such as *Sphaeropsissapinea* (Fr.: Fr.) Dyko & Sutton and *Cyclaneusma minus* (Butin) Di Cosmo, Peredo & Minter, that are important pathogens in various European countries [4].

Sphaeropsis sapinea is the most common conifer pathogen causing Sphaeropsis shoot blight or Diplodia tip blight and stem canker disease of many pine species and conifers of various ages in ornamental plantations, and natural stands in different continents [4, 5, 6]. Moreover, *S. sapinea* is well-known for its capacity to infect pine trees through various types of wounds and insect damage [5, 22]. This pathogen overwinters in infected needles, stems, forming pycnidia from which a large number of conidia are produced to infect new trees. This fungus can cause shoots blight or, vice versa, exist in a latent phase for a long period, until favorable conditions are coming for infection development [19]. Favorable climatic and environmental factors might promote spores to invade the host cells especially during shoot elongation when the fungus directly penetrates to new needles and stems, or enters into wounds through stomata [5, 19]. Water stress or drought can increase disease severity and stimulate latent infection of *S. sapinea* to become pathogenic and cause Sphaeropsis shoot blight or SSB [19] regardless of whether such stress occurs before or after the tree is infected with the fungus. Like many other Botryosphaeriaceae, *S. sapinea* is known to be opportunistic pathogen affecting conifers subjected stress factors [15, 20]. In Ukraine, pine seedlings are particularly susceptible, and this disease has caused significant losses in pine plantationsof the north-eastern part of country [2, 3].

The latent phase of *S. sapinea* has been confirmed by various authors worldwide [9, 10, 13, 19], making forest protection measurement more difficult in a timely and proactive way.Until now, identification of the *S. sapinea* as species responsible for pine plantation disease in Ukraine has been carried out in only a few areas [1, 3]. However, both *S. sapinea* and *S. scrobiculata* were detected from dead and living pine seedlings in South Africa, North America and Europe [9, 16, 17], but only *S. sapinea* was identified in Ukraine [2, 3].

The aim of this study was: i) to classify correctly the *Sphaeropsis* species occurring on different pine species growing in different environments in Ukraine; ii) to determine whether Sphaeropsis shoot blight can pose a threat to pine plantations under stress condition; iii) to detect latent infection *Sphaeropsis sapinea* in symptomless pine shoots.

Material and methods. *Sample collection and fungal DNA extraction.* Surveys were carried out in September – October 2015 at fourteen sites located in three regions of the northern (Sumy), eastern (Kharkiv) and southern (Kherson) parts of Ukraine where Scots pine (*Pinus sylvestris*) and Crimean pine (*Pinusnigra* subsp. *pallasiana*) are commonly grown. Sites were flat or gently sloped with well-drained sand soils or loamy sand. These soils are infertile and prone to drought in the summer and were covered by pine forests and small deciduous communities. The climate is continental with low to moderate precipitation level, dry and warm summers, and cold winters.

Representative samples from a number of pine forests were collected in various locations, in both urban and woodland areas, and under different climatic conditions (Table 1). Samples were taken from symptomatic shoots and asymptomatic shoots where *Sphaeropsis* spp. may have been present in latent and existing phase.

Starting at a chosen location within each site, 5 sampling plots were established at intervals of at least 20 m or more where symptomatic pine seedlings 8–12 years old were present. At each sampling point, condition of seedlings within the site was examined as either living or dead seedlings. For living seedlings, occurrence of *Sphaeropsis* tip blighted shoots was recorded and a symptoms severity rating on a 0–3 scale (Table 2) was assigned [14].

Symptomatic shoot segments were collected from each plot to examine them for the presence or absence of black, erumpent pycnidia (asexual fruiting structures) of *Sphaeropsis* species using a microscope.

For fungal isolation and DNA extraction, in total five trees were selected in each sampling area where two samples per tree (one symptomatic and one symptomless shoot) were collected, giving a total of ten shoots and needles (five symptomless and five symptomatic) from each site.

1. Characteristics of sites which were surveyed for Sphaeropsis shoot blight damage

Site	Region	Geographical origin	Latitude	Longitude	Soiltype	Host
1	Sumy	Shostka	51°51'48.6" N	33°21'47.2 "E	Strong grey loamy fine sand	*P. sylvestris*
2	Sumy	Shostka	51°52'48.6" N	33°25'05.3 "E	Strong grey loamy fine sand	*P. sylvestris*
3	Sumy	Shostka	51°45'37.4" N	33°25'03.0 "E	Strong grey loamy fine sand	*P. sylvestris*
4	Sumy	Yampil	51°58'10.8" N	33°55'31.1 "E	Strong grey loamy fine sand	*P. sylvestris*
5	Sumy	Svessa	51°58'50.0" N	33°51'57.8 "E	Strong grey loamy fine sand	*P. sylvestris*
6	Kharkiv	Vovchansk	50°11'04.4" N	36°52'10.4 "E	Plainfieldsand	*P. sylvestris*
7	Kharkiv	Chotomlya	50°00'59.5" N	36°54'00.3 "E	Plainfieldsand	*P. sylvestris*
8	Kharkiv	Zmyiv	49°38'25.8" N	36°21'03.6 "E	Plainfieldsand	*P. sylvestris*
9	Kharkiv	Botanicalgar den	50°00 N	36°13'32.3 "E	Plainfieldsand	*P. sylvestris*, *P. nigra*, *P mugo*
10	Kharkiv	MalaDanilov ka	50°04'40.0" N	36°08'27.7 "E	Plainfieldsand	*P. sylvestris*, *P. nigra*,
11	Kherson	Chulakovka	46°24'18.8" N	32°31'20.7 "E	Coarseandfines and	*P. sylvestris*, *P.nigra*
12	Kherson	Chulakovka	46°22'20.8" N	32°30'32.8 "E	Coarseandfines and	*P. sylvestris*, *P. nigra*
13	Kherson	Rybalche	46°27'31.1" N	32°14'53.9 "E	Coarseandfines and	*P. sylvestris*, *P. nigra*
14	Kherson	Heroyske	51°51'48.6" N	33°21'47.2 "E	Coarse and fine sand	*P. sylvestris*, *P. nigra*

2. Modified scale used for rating the severity of Sphaeroopsis shoot blight damage on living pine seedlings

0. None	Leader and all lateral branches free of Sphaeropsis shoot blight symptoms
1. Light	Leader free of Sphaeropsis shoot blight and canker symptoms, and exhibits no evidence of past leader damage. Only one or two lateral branch bears blighted shoot(s)
2. Moderate	More than one lateral branch bears blighted shoot(s), but 50 % of the lateral branches are symptomatic. Or, the leader was blighted in the past regardless of other shoot blight symptoms observed.
3. Severe	Regardless of whether the leader has previously been blighted, 50% of the lateral branches bear blighted shoot(s), but some live foliage is still present.

All samples were surface sterilized according to Millberg et al [11] and fungal isolation was carried out from the small sample fragments which were cut from each shoot/needles and placed in Petri dishes containing 3 % malt extract agar, MEA, and incubated at 23° C for 15 days in the dark. Pure fungal cultures were obtained from colonies morphologically classified and one representative isolate resembling the morphological group was selected.

Frozen samples were ground to a fine powder with liquid nitrogen. DNA extraction and purification was done by using Nucleo Spin® Plant II Midi kit (MACHEREY-NAGEL product). DNA quantification and quality control of the DNA samples were analyzed spectrophotometrically by way of NanoDrop ND-1000 (Wilmington, USA).

PCR with Diplodia-specific primers. The presence or absence of *S. sapinea*, *S. scrobiculata* and *S. seriata* was verified using the specific primer pairs (DpF/BotR, DsF/BotR and BotF/BotR, respectively) described by Smith and Stanosz [17, 20]. PCR was performed in a final volume of 50 µl. Each DNA sample as well as negative control was evaluated from reference strains in all reactions. A relevant amplification steps were used for the PCR with the following cycler protocol: 95°C for 5 min, 30 cycles of 95°C for 1 min, 53°C for 1 min and 72°C for 1 min with a final extension of 72°C for 5 min. PCR fragments were analyses by agarose gel electrophoresis with 0.7 g in 100 ml 1 9 Tris-boric acid-EDTA buffer (TBE) and visualized by SYBR Safe staining to detect the presence/absence of *Sphaeropsis sapinea* in samples[17]. These PCR primers may distinguish *S. sapinea* from *S. scrobiculata* and other closely related fungi.

Statistical analysis. All data were tested for adherence to the normal distribution using the Kolmogorov-Smirnov test and for homogeneity of variances using Bartlett's Test. Means of occurrence and severity data for each site were calculated using parametric methods by the HSD Tukey post hoc test with a significance level of 0.05. To evaluate the influence of site on occurrence and severity of diseases, a randomized block design, dividing the experiment on-site blocks, such that the variability within blocks is less than the variability between blocks. Mann–Whitney tests for equality of medians with Bonferroni corrections were used for pairwise comparisons of medians to examine possible effects of site. Statistical analyses were completed using R version 2.6.1 software (R Foundation for Statistical Computing, Vienna, Austria).

Results and discussion. Sphaeropsis shoot blight symptoms were observed in crowns of pine seedlings at any site, however, the incidence of SSB varied in different extent and most trees also otherwise appeared to be healthy (Table 3).

The most tree damage by Sphaeropsis shoot blight has been observed in 2015 at the site Shostka Sumy region (Table 3) where *S. sapinea* incidence was 92–97 %, owing largely to hail and windstorm in 2014 and 2015. The high severity of Sphaeropsis shoots blight at the site Shostka for the majority of seedlings and high frequency of disease on current year shoots are evidence that hail and other environmental factors might increase the infection rate as has been outlined in previous studies [9, 13, 18]. Therefore, the site did not influence the rate of seedlings with SSB nor the frequency of current year shoots blighted based on one way ANOVA tests (values of P=0.05). In contrast, a Mann-Whitney test with the Bonferroni correction (P=0.01) indicated a site effect on the disease severity (Table 3). Moreover, variability within regions showed no significant differences in severity and occurrence of pathogen between sites for Kharkiv region and significant differences within Sumy and Kherson regions between sites with water stress and hail and without ones. It can be considered to be evident that climatic factors also play key role for this disease intensity and occurrence. The mortality of seedlings, occurrence and Sphaeropsis shoots blight severity vary considerably only for both post-hail and water stress sites (Table 3). Therefore, Sphaeropsis shoot blight is a clear example of an emerging disease thereby demonstrating how environmental factors can have promoted disease appearance and spread. Many researchers also indicated *S. sapinea* as a thermophilic species (optimum temperature for growth is 30° C) and that climate events such as hail, drought or heat wave are also a likely hypothesis to explain the emergence of epiphytotic disease outbreaks [9].

3. Frequency and severity of Sphaeropsis shoot blight damage on pine seedlings of 8–12 years old frompine plantations of three regions of Ukraine

Region	Site	SSB occurrence, %			Shoot blight severity (0–3) mean±SE,[a]	Seedling mortality (%) mean±SE,[a,b]
		Total, mean ±SE,[a]	Current year shoots, mean±SE,[a]	Needles, mean±SE,[a]		
Sumy (Shostka), PHS[c]	1	96±3.1 [a]	41±2.1 [a]	68±5.2 [a]	1.05±0.01[ac]	8.2±1.1 [a]
Sumy (Shostka), PHS[c]	2	92±2.9 [a]	37±7.3 [a]	66±3.9 [a]	2.12±0.01[ac]	9.4±1 [a]
Sumy (Shostka), PHS[c]	3	97±4.2 [a]	39±3.4 [a]	71±6.1 [a]	1.52±0.15 [a]	9.9±0.8 [a]
Sumy (Yampil)	4	24±0.5 [b]	2±0.2 [b]	24±2.2 [b]	0.92±0.2 [a]	1.2±0.9 [b]
Sumy (Svessa)	5	32±1.1 [b]	7±0.1 [c]	22±3.2 [b]	0.9±0.1[a]	1.5±0.1 [b]
Kharkiv (Vovchansk)	6	34±2.1 [b]	11±0.6 [c]	31±1.6 [b]	0.65±0.08[b]	2.5±0.05 [b]
Kharkiv (Chotomlya)	7	27±2.2 [b]	7±0.2 [c]	11±2.1 [c]	0.47±0.1 [b]	3.1±0.06[c]
Kharkiv (Zmyiv)	8	19±1.1[bc]	11±0.3 [c]	14±1.1 [c]	0.45±0.08[b]	1.4±0.09 [b]
Kharkiv (Botanical garden)	9	32±2.1 [b]	19±0.2[cd]	33±2.3 [b]	0.58±0.1[b]	1.8±0.1 [b]
Kharkiv (Mala Danilovka)	10	39±3.3 [b]	10±1.1 [c]	42±2.01 [b]	0.55±0.05[b]	3.5±0.15 [c]
Kherson (Chulakovka)	11	33±1.7 [b]	12±2.1 [c]	36±3.2 [b]	1.4±0.01[ac]	5.5±0.98 [c]
Kherson (Chulakovka), WSS[d]	12	54±1.7[d]	21±1.3 [d]	41±1.5[b]	1.85±0.03[c]	5.6±0.12 [c]
Kherson (Rybalche), WSS[d]	13	52±2.3 [d]	24±2.1 [d]	44±2 [b]	1.55±0.08[c]	4.70±1.35 [c]
Kherson (Heroyske), WSS[d]	14	42±2.9 [d]	27±5.4[ad]	39±1.3 [b]	1.68±0.05[c]	7.2±0.69 [a]

[a] Mean ± standard error (SE) for total number of live and dead standing seedlings from five plots per site; values in a column with the same adjacent lowercase letters indicate site medians are not significantly different from one another based on comparison of medians (not shown) using Mann–Whitney tests with the Bonferroni correction at values of P = 0.05 if a parametric test at P=0.05 first indicated a site effect.

[b] Mean ± standard error (SE) mortality for all standing seedlings in five plots per site.

[c] –PHS — post-hail site

[d] –WSS — water stress site

Numerous previous studies have demonstrated SSB to spread as a latent pathogen in healthy pine seedlings and trees and speculated that this is a good explanation the rapid disease development and spread under stress [17, 18, 19, 20]. Furthermore, Smith et al. (2015) [17]showed that latent infection in seed cones of *Pinus* species in South Africa could be a permanent source of *S. sapinea* infection.

The presence of *Shaeropsis sapinea* in symptomatic shoots was confirmed by microscopy. Aggregate data from microscopy of symptomatic needles from symptomatic shoots showed that conidial dimensions in the sample were 27.5–41.5 (44.3) × (10.5) 12.5–15.6 µm. The identity of the *S. sapinea* from symptomless samples was confirmed by partial sequencing of the internal transcribed spacer (ITS) region at the SLU and deposited in GenBank (Accession No. KU663996). As a result of our study, *S. sapinea* have been found in symptomless plants indicating the latent stage of infection (Table 4).

4. Incidence of Sphaeropsis shoot blight damage on pine seedlings of 8–12 years old from pine plantations of three regions of Ukraine

Region	Site	Occurrence of SSB (%) (symptomatic needles and shoots infected by SSB) mean±SE, [a]	Proportion of symptomless needles and shoots infected by SSB (%) mean±SE, confirmed by PCR [a]
Sumy(Shostka), PHS[b]	1	96±3.1 [a]	9.2±1.1 [a]
Sumy(Shostka), PHS[b]	2	92±2.9 [a]	7.2±0.2 [a]
Sumy(Shostka), PHS[b]	3	97±4.2 [a]	9±0.2 [a]
Sumy(Yampil)	4	24±0.5 [b]	8.2±1.5 [a]
Sumy(Svessa)	5	32±1.1 [b]	39±1.2 [b]
Kharkiv(Vovchansk)	6	34±2.1 [b]	36±1.1 [b]
Kharkiv(Chotomlya)	7	27±2.2 [b]	32±2.1 [b]
Kharkiv(Zmyiv)	8	19±1.1 [bc]	24.2±1.1 [bc]
Kharkiv(Botanicalgarden)	9	32±2.1 [b]	28±3.1 [b]
Kharkiv(MalaDanilovka)	10	39±3.3 [b]	35±2.7 [b]
Kherson(Chulakovka),	11	33±1.7 [b]	38±3.2 [a]
Kherson(Chulakovka), WSS[c]	12	54±1.7 [d]	54±2.7 [a]
Kherson(Rybalche), WSS[c]	13	52±2.3 [d]	54±3.1 [d]
Kherson(Heroyske), WSS[c]	14	42±2.9 [d]	52±5.8 [d]

[a] Mean ± standard error (SE) for total number of seedlings from five plots per site; values in a column with the same adjacent lowercase letters indicate site medians are not significantly different from one another based on comparisons of medians (not shown) using Mann–Whitney tests with the Bonferroni correction at values of P=0.05 if a parametric test at P=0.05 first indicated a site effect.

[b -] – PHS — post-hail site

[c] –WSS — water stress site.

We also consider the probable presence of latent infections caused by *S. sapinea* in *P. sylvestris* resulting in decline and mortality following hail or stress damage. For some sites, such as Kherson (all sites), Sumy (Svessa site) and Kharkiv (Vovchansk site), frequency of *S. sapinea*in symptomless shoots was even exceeding occurrence of symptomatic Sphaeropsis shoots blight (Table 4).

Moreover, the pine engraver beetle (*Ips acuminatus* Gyll. (Coleoptera: Curculionidae) and other bark beetles are considered plausible vectors of *S. sapinea* as they could transmit the pathogen during maturation and larvae feeding. As *Ips acuminatus*, one of the most common bark beetles in Ukraine was found to be in strong association with *S. sapinea*[7] that could increase the potential risk of spread of the pathogen and disease throughout the country.

Conclusion. For all sampling plots at all three sites, significant differences in disease intensity and occurrence have been observed in 2015, indicating that all stands had been blighted in the past and environmental factors (hail, wind, wounds, and water stress) may increase the severity of Sphaeropsis shoot blight triggering latent infection. Ultimately, we consider the possibility that *Spheropsissapinea* may be present in the form of latent infections in the shoot tissue or cones of *P. sylvestris* trees, and that these infections may promote Spheropsis shoot blight development associated with hail wounds and water stress.

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