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Identification and virules of *Sphaeropsis* tip blight (*Sphaeropsis sapinea*) on *Pinus* spp. in Istanbul and Bursa parks

Filiz ÜNAL[1*], Ercan KOCA[1], Ayşe AŞKIN[1], İlker KURBETLİ[2], Kamil SARPKAYA[3]

[1]Plant Protection Central Research Institute, Ankara, Turkey.
[2]Batı Akdeniz Agricultural Research Institute, Antalya, Turkey.
[3]Pistachio Research Institute, Gaziantep, Turkey.
*Corresponding author: filiz.unal@tarim.gov.tr

Abstract: *Sphaeropsis sapinea* tip blight is one of the most destructive fungal diseases of pine trees in the world. It causes shoot blight, canker, collar rot, root disease and blue stain of *Pinus* spp. As a result of surveys performed in the parks, recreation areas and groves in Istanbul and Bursa provinces in January, June and October 2015, it was observed that the agent especially causes disease on *Pinus* spp. The disease was observed as dead, brown needles at the tips of branches, shoot blight and cankers on pine stems and branches. Twenty eight diseased pine samples were collected from parks, recreation areas and groves. Totally 15 *Sphaeropsis sapinea* isolates were obtained. Cultural and morphological characteristics of the isolates were determined using cultures grown on potato dextrose agar (PDA). Cultures on PDA were initially white with profuse aerial mycelia that after one week turned grey to blackish. Identification of the pathogen was based on morphological characteristics of the conidia and DNA sequence analysis. Genomic DNA was extracted and the internal transcribed spacer (ITS) region of the rDNA was amplified using primers ITS1 and ITS 4. Pathogenicity tests were made with twig inoculation method. All isolates formed lesions on twigs.

Keywords: *Sphaeropsis sapinea*, Tip blight, Molecular, *Pinus* spp.

Introduction

Sphaeropsis sapinea (Fr.) Dyko & B. Sutton (Syn: *Diplodia pinea*) is a latent, opportunistic conifer pathogen on *Pinus* spp. The fungus affects both young and old trees causing shoot blight, dead top, sap stain, root disease, and cankers on stems and branches (Brookhouser and Peterson, 1971; Peterson, 1977). It mostly attacks *Pinus* species but also *Abies* Mill., *Chamaecyparis* Spach., *Cupressus* L., *Larix* Mill., *Picea* A. Dietr., *Pseudotsuga* Carriere and *Thuja* L. (Sinclair and Lyon, 1987; Swart et al., 1987; Chou and MacKenzie, 1988; Nicholls and Ostry, 1990; Stanosz et al., 2001; Wingfield and Knox-Davies, 1980; Palmer and Nicholls, 1985; Rees and Webber, 1988; Stanosz and Cummings-Carlson, 1996).

The fungus was first seen in Austria (*Pinus nigra*) and Scottish (*Pinus sylvestris*) in the world (Peterson, 1981). It was first reported in Kahramanmaraş forests on *P. nigra, P. elderica* and *P. brutia* in Turkey by Sümer (Sümer, 2000). In 2007, the pathogen was noted on *P. brutia* in Isparta, Turkey (Doğmuş-Lehtijärvi et al., 2007). Several years later it was found on *Pseudotsuga menziesii* (Kaya et al., 2014) and on *P. nigra* and two *Pinus sylvestris* in İzmit province (Doğmuş-Lehtijärvi et al., 2014). Although *S. sapinea* has been reported on forests in Turkey, there is not record about the development of the disease in Turkish parks.

The aim of this study, identification of *Sphaeropsis sapinea* on *Pinus* spp. in Istanbul and Bursa Parks and detection of their virulens on *Pinus nigra* twigs.

Materials and Methods

Survey and isolation: As a result of surveys performed in the parks, recreation areas and groves in Istanbul and Bursa provinces in January, June and October 2015, symptoms of the disease were observed on *Pinus* spp. The disease was observed as dead, brown needles at the tips of

branches, shoot blight and cankers on pine stems and branches. Twenty eight diseased pine twig and cone samples were collected from parks, recreation areas and groves. For isolation of the fungi, small pieces of discolored tissues were excised from lesion margins, surface sterilized in 0.5% sodium hypochlorite for 1-2 min, rinsed and dried on sterilized filter paper, then placed on potato dextrose agar (PDA, Difco) plates containing 50 mg/l streptomycin sulfate. Plates were incubated at 25°C for 5 to 7 days. Hyphal tips of *Sphaeropsis*-like fungi were transferred to PDA and placed under fluorescent light (12 h/day). Twigs and cones were investigated for the presence of pycnidia using a stereomicroscope. Pycnidia were transferred onto PDA and incubated at 25°C for 5-7 days.

Morphological and molecular identification: Morphological features of the conidia collected from the pycnidia and cultural characteristic of the isolates grown on PDA were recorded. For molecular identification, approximately 300 mg mycelium were harvested and ground with liquid nitrogen in a sterile mortar for DNA extraction. Genomic DNA was extracted using a Qiagen DNeasy ® Plant Mini Kit, as specified by the manufacturer, and stored at -20°C prior to use. PCR reaction mixtures and conditions were modified from previous studies (Aroca and Raposo, 2007; Cobos and Martin, 2008). The reaction mixtures of PCR, a final volume of 50 µl, contained 5µl of 10X buffer [75 mM Tris HCl, pH 9.0, 50 mM KCl, 20 mM $(NH4)_2SO_4$], 2 µl of 5 µM each primers, 5 µl of 1.5mM MgCl2, 2 µl of 10 mM deoxynucleoside triphosphates (dNTPs), 1 U Taq polymerase (Fermantas), 5 µl of DNA template for each reaction and 5 µl of bovine serum albumin (BSA: 10 mg/ml). DNA amplifications were carried out in a Techne TC-5000 thermal cycler by the following program: 94 C for 2 min, followed by 34 cycles of (1) denaturation (94˚C for 30 s), (2) annealing (60˚C for 30 s) and (3) extention (72˚C for 30 s), and a final extension step 10 min at 72˚C. The ITS region of the isolate was amplified using the universal primers ITS-1 (5' TCCGTAGGTGAACCTGC GG 3') and ITS-4 (5' TCCTCCGCTTATTGA TATGC 3') (White et al., 1990). The PCR products were separated in 1.5% agarose gels stained with ethidium bromide, and visualized under UV light. Sequence analysis was performed by GENOKS (Gene Research and Biotechnology Company, Ankara, Turkey).

Pathogenicity Tests: In order to determine the virulens of isolates, *P. nigra* twigs were used collected in the survey areas. The needles were removed and the twigs were cut to about 20 cm in length. Fifteen isolates were grown on PDA at 24°C in the dark for one week. The inoculation point was cleaned with 70% (v/v) ethanol. Using a sterilised cork borer, a circular 4-mm wound was made by removing the bark. An agar plug colonised by mycelium was attached into the wound by wrapping Parafilm® around the twig. Sterile agar plugs were similarly applied to control twigs. Three twigs were used for each isolate. After a 4-week incubation in a growth chamber at 21°C, lesions lengths were measured. Re-isolations were made using PDA medium to confirm *S. sapinea* as the cause of the lesions. Analysis of Variance (ANOVA) was performed on the lesions length data using the SPPS GLM procedure (SPSS Inc., Chicago, USA) and statistical differences among mean values were assessed using Duncan's Multiple Range test (P<0.05) (Doğmuş-Lehtijärvi et al., 2014).

Results and Discussion

As a result of surveys performed in the parks, recreation areas and groves in Istanbul and Bursa provinces, 28 diseased pine samples were collected from parks, recreation areas and groves. Totally 15 *S. sapinea* isolates were obtained. Cultural and morphological characteristics of the isolates were examined using cultures grown on potato dextrose agar (PDA).

Conidia were ovoid, rounded at the apex, brown to dark brown, usually 0-1 septate, thick-walled (30-45×10-16 µm) (n=100) on light microscope, which was within the range for the fungus. Colony morphology was also as described by de Wet et al. (2000). Initially a floccose white mycelium developed; the colour turned first dark grey in a few days, and then gradually black from the centre towards the margin (Fig. 1).

The resulting sequences were compared to other *Sphaeropsis* sequences and were 98 to 99% identical to other *S. sapinea* sequences in the GenBank. *S. sapinea* was previously detected in forests in Marmara Region by Kaya et al. (2014) and Doğmuş-Lehtijärvi et al. (2014).

In pathogenicity tests, the tested isolates caused dark brown-to-black discolouration in *P. nigra* twigs, measuring on average (±SD) 15.2 (±1.2) cm, in length of xylem of the twigs. The pathogen was successfully re-isolated from symptomatic twigs. There were no

Figure 1. Colony appearance and conidia of *Sphaeropsis sapinea*.

Table 1. Pathogenicity of *S. sapinea* isolates on *Pinus nigra* twigs.

Isolates	Means	Standart	Pathogenic
B8	19.50	±0.50	a
İs 6	18.80	±1.31	ab
B 2	18.60	±0.53	abc
İs 1	18.33	±0.38	abc
B 9	18.08	±1.08	abc
B 6	17.70	±0.52	abc
İs 2	17.40	±0.43	abcd
B 4	16.60	±1.61	bcde
B 7	16.16	±1.37	cde
İs 5	15.09	±0.77	def
İs 3	14.60	±1.51	efg
B 1	13.06	±1.99	fg
İs 4	12.20	±3.63	g
B 3	12.08	±1.88	g

*average disease indices followed by the same letter reflect frequency distributions that do not differ significantly from one another ($P<0.05$).

differences in lesions length among the isolates. Similarly, Chou (1976, 1987) and Doğmuş-Lehtijärvi et al. (2014) found no differences among New Zealand isolates obtained from *P. radiata*.

In the pathogenicity tests, tested isolates caused dark brown-to-black discolouration and extended from 19.50 cm to 12.08 cm in vascular system on *P. nigra* twigs. Totally, 14 isolates were pathogenic and only one isolate gave no symptom on experimental plants and accepted as non-pathogenic. Isolates were statistically groupped in Table 1. The pathogen was successfully re-isolated from symptomatic twigs.

In conclusion, *S. sapinea* was identified on *Pinus* spp. in Bursa and İstanbul parks, recreation areas and groves. For many years this fungus was recorded only from Turkish forests but with this study, it was also reported from the parks in Marmara Region. Regarding this situation, there is a risk for *S. sapinea* to spread to the other provinces of Turkey. It is necessary to conduct surveys in other province parks and restrict or prevent the spread of this pathogen.

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