

## Occurrence and Genetic Similarity of *Diplodia pinea* on Shoots and Cones in Seed Orchards of *Pinus* spp. in North-Western Turkey

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### Abstract

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*Diplodia* shoot blight disease can cause significant damage on coniferous trees and be particularly injurious to cones, which reduces the amount of seed production and germination. We investigated the disease severity and genetic variation of *Diplodia pinea* in one *Pinus nigra* and two *P. sylvestris* seed orchards. Disease surveys were carried out in İzmit (Marmara region, Turkey) in May 2012. Symptomatic shoots and cones were examined for the presence of pycnidia. Cultural and morphological characteristics of the isolates were studied using cultures grown on potato dextrose agar (PDA). Based on morphological characteristics and results using species specific primers, the pycnidia on shoots and cones were identified as *D. pinea*. In addition, Random Amplified Microsatellite Sequence (RAMS) analyses indicated that there was a single genet of *D. pinea* which caused the disease in the seed orchards. All of the 60 sampled trees were found to be infected by the fungus. There were differences in disease severity among the stands.

**Keywords:** diplodia shoot blight; pine; RAMS; disease severity

*Diplodia pinea* (Desm.) Kickx is a latent, opportunistic conifer pathogen which causes the disease commonly known as Diplodia shoot blight (ELDRIDGE 1961; SWART & WINGFIELD 1991; STANOSZ *et al.* 1997). The fungus affects both young and old trees causing shoot blight, dead top, sap stain, root disease, and cankers on stems and branches (BROOKHOUSER & 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 & LYON 1987; SWART *et al.* 1987; CHOU & MACKENZIE 1988; NICHOLLS & OSTRY 1990; STANOSZ *et al.* 2001; WINGFIELD & KNOX-DAVIES 1980; PALMER & NICHOLLS 1985; REES & WEBBER 1988; STANOSZ & CUMMINGS-CARLSON 1996).

Although *D. pinea* has been already reported in Turkey (GÜNAY 2001), little is known about the development of the disease in Turkish forests. Some of the most severe damages have occurred in southern Turkey. The pathogen was noted on dead twigs and canker samples of *P. elderica* and *P. brutia* in Kahramanmaraş in 2005. Several years later it was found on *Pseudotsuga menziesii* in İzmit province (KAYA *et al.* 2014). Studies carried out in the western part of the Taurus Mts. in Isparta province, located in the Mediterranean part of Turkey, showed that *D. pinea* was the most common cause of the shoot blight of *P. brutia* Ten. (DOĞMUŞ-LEHTIJÄRVI *et al.* 2007).

In 2013, we investigated one *P. nigra* and two *P. sylvestris* seed orchards infested by *D. pinea* in

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İzmit-Kerpe in north-western Turkey in order to: (i) confirm the occurrence of *D. pinea* by molecular techniques, (ii) assess the genetic variation among isolates obtained from these stands, (iii) estimate the disease severity of Diplodia shoot blight, and (iv) test the ability of *D. pinea* isolates to colonize excised pine twigs.

## MATERIAL AND METHODS

**Study site and disease severity.** Surveys were conducted in May 2012 in one *P. nigra* and two *Pinus sylvestris* seed orchards that were infested by *D. pinea*. The orchards were located 600 m a.s.l. in Kerpe Research Forest in İzmit province in the Marmara region.

Sixty trees were sampled in transects, in which every fifth tree was investigated for the presence of *D. pinea*. For each sampled tree the disease severity was rated after dividing the living crown into three parts: the lowermost 20%, the middle 50%, and the uppermost 30% of the crown height. These lower, middle, and upper parts of the crown were rated 0–30, 0–50, and 0–20%, respectively (i.e. the rating was adjusted to emphasize the infections in the lower part of the crown). The disease severity in the whole crown (0–100%) was calculated as the sum of the rates of the crown parts. The percentage data was subjected to arcsine transformation prior to statistical analysis. Symptomatic shoots and mature, open cones remaining on shoots were collected from each sampled tree. In addition, recently fallen, opened (but not weathered) cones were collected from the ground.

**Morphological and molecular identification.** Shoots and cones were investigated for the presence of pycnidia using a dissecting microscope. Pycnidia were transferred onto potato dextrose agar (PDA; Merck KGaA, Darmstadt, Germany) (39.1 g/l of potato-dextrose agar) and incubated at 24°C for 5 days. Sixty isolates, twenty from each stand, were obtained. Morphological features of the conidia collected from the pycnidia and cultural characteristic of the isolates grown on PDA were recorded.

Agar plugs of growing mycelium of three representative isolates were placed into 1.5 ml micro centrifuge tubes containing 500 µl of potato dextrose broth (PDB; Sigma-Aldrich, St. Louis, USA). The cultures were incubated for 5 days thereafter DNA was extracted from the cultures using the method described by SMITH and STANOSZ (1995). The DNA was amplified using the primer pair DpF (5'-TTATATATCAAACCTATGCTTTGTA-3') and BotR (5'-GCTTACACTTTCATTTATAGACC-3') (SMITH

& STANOSZ 2006). The amplification products were separated by electrophoresis in gels containing 1% (w/v) of agarose.

**RAMS analyses.** Genetic variation among the isolates was determined using multilocus genotyping with the RAMS-primers TCG (VDH (TCG)<sub>5</sub>) and DDB(CCA)<sub>5</sub> where: B = G/T/C, D = G/A/T, H = A/T/C, and V = A/C/G (HANTULA *et al.* 1996; primers synthesised by IonTek, Istanbul, Turkey). Thirty isolates were used for the analysis (ten from each stand). DNA was extracted as described above. The DNA samples were denatured at 95°C for 10 min, after which 37 cycles of amplification were carried out (30 s denaturation at 95°C, 45 s annealing at a primer dependent temperature, and 2 min extension at 72°C), followed by 7 min incubation at 72°C (HANTULA *et al.* 1996). Amplification products were separated by electrophoresis on gels containing 1% (w/v) of agarose (Bio Basic Inc., Markham, Canada) and SynerGel (Diversified Biotech, Boston, USA) in TAE buffer and length of the products was estimated using DNA molecular size markers with 100 bp repeats.

**Excised twig inoculation.** The ability of the isolates to colonise excised, one-year-old twigs collected in the field from single mature *P. sylvestris* and *P. nigra* trees was tested. The needles were removed and the twigs were cut to about 20 cm in length. Nine isolates (three from each stand) 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. Five twigs were used for each isolate-host combination. Each of the nine isolates was inoculated into one twig of each host species and placed randomly in a box used for incubation. Totally five replicate boxes (9 × 2 × 5 = 90 twigs) were used. 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 *Diplodia pinea* as the cause of the lesions. Analysis of Variance (ANOVA) was performed on the lesions length data using the SPSS GLM procedure (SPSS Inc., Chicago, USA) and statistical differences among mean values were assessed using Duncan's Multiple Range test ( $P < 0.05$ ).

## RESULTS AND DISCUSSION

**Disease severity.** All the 60 sampled trees were found to be infected by *D. pinea*. The symptoms were

Table 1. Severity of Diplodia shoot blight symptoms in the surveyed stands

	<i>Pinus sylvestris</i>		<i>Pinus nigra</i>	Mean
	stand 1	stand 2	stand 3	
Upper part of crown (0–20%)	3.3 <sup>b</sup> ± 0.98 <sup>ab</sup>	2.3 <sup>b</sup> ± 0.67 <sup>b</sup>	7.6 <sup>a</sup> ± 1.17 <sup>a</sup>	4.4
Middle part of crown (0–50%)	13.9 <sup>b</sup> ± 2.66 <sup>b</sup>	11.2 <sup>b</sup> ± 1.65 <sup>c</sup>	29.2 <sup>a</sup> ± 2.10 <sup>a</sup>	18.1
Lower part of crown (0–30%)	16.3 <sup>b</sup> ± 2.10 <sup>ab</sup>	10.8 <sup>c</sup> ± 1.42 <sup>b</sup>	21.7 <sup>a</sup> ± 1.40 <sup>a</sup>	16.3
Total (0–100%)	33.5 <sup>b</sup>	24.3 <sup>c</sup>	58.5 <sup>a</sup>	38.8

Means within a row followed by different letter are significantly different ( $P < 0.05$ ) based on Duncan's Multiple Range test

scattered in all parts of the tree crowns. Diseased trees showed symptoms, such as shoot blight and dieback, similar to those reported by MARESI *et al.* (2007). In addition to symptoms of Diplodia shoot blight, loss of young needles was observed and attributed to drought in the stand.

There were differences in disease severity among the stands (Table 1). *P. nigra* stand had the highest average whole crown disease severity ( $P < 0.05$ ). In *P. sylvestris* stands, the average disease severity in the upper and the lower part of the crown did not differ significantly among the stands. However, there was a statistically significant difference in the average disease severity for the whole crown also for *P. sylvestris* stands.

**Morphological and molecular identification.** All the 60 isolates obtained from cones and shoots were identified as *D. pinea*. Conidia were brown to dark brown, thick-walled (10–24 × 42–20 μm;  $n = 100$ ), which was within the range for the fungus (DE WET *et al.* 2000). *D. pinea* 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.

**Molecular identification and RAMS analyses.** The primer pair DpF/BotR amplified an approximately 700–750 bp fragment from the isolates and confirmed their identity as *D. pinea*. Amplification with each of the two RAMS primers produced five DNA fragments ranging from 350 bp to 870 bp in length. These primers revealed six non-variable bands. Gene copies, haplotypes, and loci numbers were the same in all stands.

The results suggested that the trees were colonised by a single *D. pinea* genet. This is in accordance with the results of other studies of the fungus showing that genetic variation between *D. pinea* populations is low even at the global scale. STANOSZ *et al.* (1999) found high degree of similarity among isolates of *D. pinea* with the aid of RAPD marker and isozyme analysis.

BURGESS *et al.* (2004) showed with SSR markers that six populations of *D. pinea* (in north-east America, Michigan, Central Europe, New Zealand, west Australia, and South Africa) exhibited moderate to very low gene diversities with little population differentiation. The Central European population of *D. pinea* was clonal with only three genotypes among 28 isolates.

In our study, the two RAMS markers were not enough to detect any genetic variation between *D. pinea* isolates obtained from the two different host species. Further analyses using SSR markers or other microsatellite markers are needed to clarify the population genetics and possible host specialisation of *D. pinea*.

**Excised twig inoculation.** The tested isolates caused dark brown-to-black discolouration in the *P. sylvestris* and *P. nigra* twigs, measuring on average (± SD) 17.3 (± 0.6) cm and 16.4 (± 1.2) cm, respectively, in length of xylem of the twigs. The pathogen was successfully re-isolated from symptomatic twigs. There were no differences in lesions length among the isolates. Similarly, CHOU (1976, 1987) found no differences among New Zealand isolates obtained from *P. radiata*.

In conclusion, *D. pinea* had some serious effects on *P. sylvestris* and *P. nigra* seed orchards of the Kerpe Research Forest and could be the most common causal agent of shoot blight and seed reduction in the Kerpe Research Forest in İzmit. The symptoms have increased over the past 3 years since its first detection there, reduction in seed production in these orchards has occurred. Although the site is located in a region with humid climate and approximately 800 mm of annual rainfall, there have been periods of drought in each of the past three years. It is possible that drought stress predisposed the trees to be attacked by *D. pinea*, as has been reported elsewhere (DOĞMUŞ-LEHTIJÄRVI *et al.* 2007).

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