Detection of *Diplodia pinea* in Asymptomatic Pine Shoots

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Abstract – *Diplodia pinea* is a fungal pathogen that causes tip blight shoot on several conifers trees. During the last few years the occurrence of this fungus in symptomless pine shoots, has been investigated on Austrian pine (*Pinus nigra* A.) pinewoods located in Central and Northern Italy. Fungal detection from apparently healthy samples was performed by using both isolation of pine tissue on agarized media but also by the real-time PCR. Differences between two sampled pinewoods were found showing a different behaviour of the fungus in the host.

Conifers / fungi / latent phase / real-time PCR / *Sphaeropsis*

Kivonat – A *Diplodia pinea* kimutatása tünetmentes *Pinus* hajtásokban. A *Diplodia pinea* kórokozó gomba a fenyőfélék hajtáspusztulását okozza. Az utóbbi években e gomba tünetmentes előfordulását vizsgáltuk feketefenyő (*Pinus nigra* A.) erdőkben, Közép- és Észak-Olaszországban. A látszólag egészséges mintákban a gombát a fenyők szöveteiből való kitenyésztéssel és real time PCR módszerrel mutattuk ki. A gomba eltérő viselkedését mutató különbségeket találtunk két megmintázott fenyőerdő között.

Fenyők / gombák / latens fázis / real-time PCR / *Sphaeropsis*

1 INTRODUCTION

Diplodia pinea (= *Sphaeropsis sapinea* (Fr.:Fr.) Dikko & Sutton) is a fungus with a world-wide distribution (Stanosz et al.1996) responsible of shoot dying of pines. Two *S. sapinea* morphotypes, initially designated A and B, were indicated as two different species: *Diplodia pinea* for the A morphotype and *Diplodia scrobiculata* for the B morphotype (de Wet et al. 2003). They basically differ for conidia and colony morphology and aggressiveness against host plants (Smith-Stanosz 1995, Hauser et al. 1999, de Wet et al. 2002). The two species can be differentiated more clearly using molecular techniques (Smith – Stanosz 2006, Zhou – Stanosz 2001).

In Italy *D. pinea* occurs on some Mediterranean species of *Pinus* along the peninsula, like *P. halepensis*, *P. pinaster*, *P. pinea*, and *P. nigra*. In this country the main damages are recorded on *Pinus nigra* plantations, especially those present in Northern Italy where the fungus is particularly injurious, causing tip blight and progressive dying of trees (Maresi et al. 2002). On pine the fungus, occasionally vectored by insects (*Tomicus* sp.), causes also blue stain of wood

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(Sabbatini Peverieri et al. 2006). Other *D. pinea* disease occurs on cones, related with the cone bug, *Gastrodes grossipes* (Feci et al. 2002). Along the Tyrrhenian coast of peninsula, the main damages on cones have also an economic impact on edible seeds production on *P. pinea* (Vagniluca et al. 1995).

Studies on the environmental conditions enhancing the outbreak of the disease on pine plantations underlined the influence of water stress on the host susceptibility (Stanosz et al. 2001, Paoletti et al. 2001). *D. pinea* is able to live for long time inside the host tissue in latent phase, without any visible symptoms, until climatic and environmental factors induce the fungus to invade host cells and cause death of the tissues (Stanosz et al. 2001).

Massive presence of latent infections may explain the ineffectiveness of pruning and spraying in controlling the disease (Flowers et al. 2001). Is not known how long *D. pinea* might persist in symptomless trees, but was observed that the fungus can rapidly become pathogenic after water stress condition for host (Stanosz et al. 2001).

The occurrence of *D. pinea* in host tissue have been described by using classical approaches, such as plating pine samples on agar media (Stanosz et al. 2001; Flowers et al. 2001), but also with molecular techniques (Flowers et al. 2003; Luchi et al. 2005) that revealed sensitive tools for the detection of this fungus.

2 MATERIALS AND METHODS

The study was carried out in two Austrian pine (*Pinus nigra* Arn.) plantations located in Tuscany (Montesenario, Florence; 700 m a.s.l.) and South Tyrol (Val D'Adige, Trento; 600 m a.s.l.). Eighteen trees were selected from each pinewood. For each tree one symptomless shoot was cut, from the lower part of the crown and the apical portion was used to detect *D. pinea* in host tissue. Each sample was longitudinally split in two portions and the fungal occurrence was detected by isolation on agarized media (as number of fragments colonized by *D. pinea*) and by using real-time PCR (Maresi et al. 2007). For each sampling sites 10 needles per shoot were processed for fungal detection: 5 were used for isolation and 5 for real-time PCR, according to the method already used by Maresi et al. (2007). The fungal presence was evaluated as percentage of fragments colonized by *D. pinea*, and colonies were identified according to Luchi et al., (2007). After using real-time PCR, the fungal picograms were expressed as pg DNA *D. pinea*/ μg total DNA.

3 RESULTS

The occurrence of *D. pinea* was detected in symptomless pine shoots in Trentino and Tuscany pinewoods with significant differences ($p=0.014$) between two sites.

Molecular approach showed a total of 17 out of 18 positively colonized shoots in Montesenario (*D. pinea* DNA rangedg from 0.01 to 2.5 pg), and 14 out of 18 (fungal DNA ranged from 0.02 to 1.4×10^3 pg) in Val d'Adige.

Results of isolation showed that the number of colonised shoots was lower than those obtained by real-time PCR. After plating method only 3 samples out of 18 collected in Montesenario (1 fragment for each one) and 10 out of 18 from Val d'Adige (mean: 9 fragments per shoot), resulted colonised by the fungus.

Some relationship between amount of fungal DNA colony presence after plating was also recorded. No fragments were colonized when the amount of *D. pinea* DNA was lower than 3 pg. This data was recorded in 94% from shoots collected in Montesenario and 44.4% from Val D'Adige. Occurrence of *D. pinea* was always negative both after isolation or real-time PCR.

4 DISCUSSION

In this study the use of both cultural and molecular methods for fungal detection, showed the presence of *D. pinea* in symptomless Austrian pine shoots, with differences between the two sampling areas. In the place of Trentino the amount of *D. pinea* was significantly higher than in the site from Tuscany.

Although is known that the fungus can rapidly become pathogenic after water stress condition that affect the host (Stanosz et al. 2001), is still unknown how long *D. pinea* might persist in the shoots before to produce symptoms.

In a recent work Maresi et al. (2007) showed that the frequency of *D. pinea* in symptomless pine tissue was positively correlated with Normalized Insolation index (NIi), considered as the solar radiation that trees receive every years.

Results from this study may explain further aspects of fungal behaviour. In Tuscany the occurrence of the fungus detected both in terms of colonized fragments but also as fungal DNA, was high as frequency on samples but low in term of quantity, indicating that the latent phase was prevalent. On the contrary, the bigger amount of fungal DNA detected in Trentino give the idea that the fungus although still on asymptomatic shoots, is moving from latent phase, to the parasitic phase.

For the last aspect the use of a sensitive tool, able to detect the latent phase in symptomless tissue, and their relationship with environmental parameters may be useful to predict the outbreak of *Diplodia* blight infection in pine forests.

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