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Leptoglossus occidentalis and *Diplodia pinea*: a new insect-fungus association in Mediterranean forests

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Summary

Leptoglossus occidentalis, an insect native to North America, was inadvertently introduced into Italy about 1999. The insect damages the cones of conifer trees, especially *Pinus pinea* (Italian Stone pine). *Pinus pinea* is also affected by *Diplodia pinea*, a fungus native to Italy, which is becoming an increasing threat because pine trees are becoming more susceptible to it as a consequence of global warming. Because the insect and the fungus both have the pine cones as a common habitat, a possible interaction between them has been postulated. The aim of this study was to ascertain whether *L. occidentalis* and *D. pinea* interact on *P. pinea* cones. The interaction was studied using real-time PCR on a group of naturally infected insects collected from a forest, and a group raised in the laboratory and artificially inoculated with *D. pinea* conidia. Molecular analysis showed that *D. pinea* DNA occurred on both naturally infected and inoculated insects, but with significant differences between the two groups. The rapid and sensitive molecular technique made it possible to detect *D. pinea* DNA on the bodies of the insects, and to show that the native *D. pinea* occurred on the exotic insect.

1 Introduction

In the last few years, global change has affected forest ecosystems and has lead to modifications in the interactions between many plants, pathogens and insects (Sturrock et al. 2011). As a result, many exotic species introduced from abroad are beginning to have an effect on existing native species, including pathogenic fungi (Loo 2009). For this reason, studies on plant diseases also focus on exotic insects, which may be the vectors of new exotic pathogens, or which may prove to be better vectors for native pathogens (Lovett et al. 2006).

In Italy, *Diplodia pinea* (Desmaz.) J. Kickx is a native fungal pathogen of conifers, especially Mediterranean pines, on which it causes a tip-blight of the crown (Swart and Wingfield 1991; Stanosz et al. 1996). It is particularly injurious to the cones of Italian stone pine (*Pinus pinea* L.), where it reduces the amount of edible seeds produced (Vagniluca et al. 1995; Santini et al. 2008).

Tip-blight infections from *D. pinea* are primarily caused by the conidia (Feci et al. 2003), but latent infections on symptomless trees also occur (Flowers et al. 2003; Maresi et al. 2007). In the case of latent infections, the disease does not become manifest until water stress weakens the tree (Stanosz et al. 2001; Slippers and Wingfield 2007). Global warming leads to changes in the rainfall pattern as well as higher temperatures, with more prolonged and frequent drought events that induce stress in plants (Desprez-Loustau et al. 2006). These changes impair the physiology of the host tree, and predispose it to attacks from weak pathogenic fungi that would not be able to damage thriving and unstressed trees. As a further consequence of global warming, a disease may expand to new areas where it did not occur before (Walther et al. 2009). In this context, *D. pinea* has invaded both northern Europe and South Africa (Slippers and Wingfield 2007; La Porta et al. 2008).

Over the last few years, the monitoring of fungi in plants has become more reliable through the advent of molecular techniques. Of these, real-time quantitative PCR is a rapid and sensitive method to detect and quantify small amounts of fungal DNA (Orlando et al. 1998; Schena et al. 2004). This approach is so sensitive that it is able to detect fungi in plants before visible symptoms appear (Luchi et al. 2005a).

The spread of *D. pinea* mainly occurs when its conidia are dispersed by rain and wind during the growing season (Palmer et al. 1988). However, various insects also disperse the conidia. The cone bug *Gastrodes grossipes*, for example, which has the same habitat as *D. pinea*, spreads the conidia between the cones of *Pinus nigra* (Feci et al. 2002). Other insects, such as the bark beetle *Ips pini*, are vectors of *D. pinea* on Austrian pine (Whitehill et al. 2007).

Over the last few years, special attention has been devoted to exotic insects and the ecological impact they are having on native communities (Kenis et al. 2009). And in fact, an increasing number of associations between exotic insects and native fungi have been found in forest ecosystems. *Dendroctonus valens*, introduced to China from North America, has become associated with native Chinese ophiostomatoid fungi, particularly *Leptographium procerum* (Lu et al. 2009). *Tetropium fuscum*, an insect introduced to Atlantic Canada from Europe, has formed an association with *Ophiostoma piceae* and *Pesotum fragrans*, both native to Canada (Jacobs et al. 2003).

In northern Italy, an exotic insect, *Leptoglossus occidentalis* Heidemann (the western conifer seed bug; Hemiptera; Heteroptera; Coreidae), originally from the United States, was reported for the first time in 1999 (Bernardinelli and Zandigiacomo 2001). This species is now widespread in almost the entire Italian peninsula and is rapidly spreading to countries in Southern and Western Europe (OEPP/EPPO 2010).

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Leptoglossus occidentalis feeds by sucking the sap of the seeds in the cone endosperm, causing the seeds to be aborted (Bates and Borden 2005). In Italy, the insect lowers the production of *P. pinea* seeds, which is an important source of income (Roversi et al. 2011). It occupies the same habitat as *D. pinea*. The co-occurrence of insect and fungus on pine may cause considerable damage to the tree, so that it is vital to have an understanding of the fungus and of the disease outbreak mechanism.

The aim of this study was to determine whether there is an association between the native *D. pinea* and the exotic *L. occidentalis*. A real-time PCR assay was devised to detect *D. pinea* both on the bodies of the insect and on the symptomless *P. pinea* cones that are the insect's main food source.

2 Materials and methods

2.1 Sample material

Thirty-six adult specimens of *L. occidentalis* (24 laboratory-grown and 12 from a natural forest) and 10 apparently healthy *P. pinea* pine cones were examined to detect *D. pinea*.

The 24 laboratory insects came from an established laboratory colony (CRA-ABP, Firenze, Italy). They were fed on Austrian pine seedlings, shoots and seeds of Douglas-fir (*Pseudotsuga menziesii* (Mirbel) Franco), and divided into two groups each receiving a particular means of inoculation, plus a control group:

1 Twelve insects were inoculated with a conidial suspension of *D. pinea* (CS group). Each insect received 200 μl of the suspension, applied with a micropipette. The suspension was obtained from typical pycnidia of *D. pinea*, growing on *P. pinea* cones from the Regional Natural Park of Migliarino – San Rossore (Pisa, Italy; N43°42′49.13″, E10°19′26.52″; 11 m a.s.l.). The suspension was adjusted to give 8×10^2 conidia per insect.
2 Seven insects were inoculated by allowing them to crawl on pine cones infected with *D. pinea*, which adhered to their bodies (PCI, Pine cone inoculation group). Two-year-old *P. pinea* cones bearing mature pycnidia were placed in small open plastic boxes containing moist filter-paper, and the boxes were placed in the insect cages so that the insects would crawl over the cones and be inoculated with the conidia spilling from the pycnidia. After 10 days, these insects were collected.
3 A control group of five insects was left uninoculated and was washed (UC, Uninoculated control group) after vortexing in sterile water for 2 min.

The twelve insects from a natural habitat were collected in October 2009 in the forest of Vallombrosa, near Florence, Italy (N43°43′54.75″, E11°32′59.96″; 1021 m a.s.l.). Seven of these insects were used directly for DNA extraction (VF, Vallombrosa forest group), while the remaining five were first vortexed in sterile water for 2 min and then the DNA was extracted from them (VFW, Vallombrosa forest washed group). The water with which these five insects were washed (WW, washing water) was used to determine the amount of fungal DNA adhering to the surface of each insect.

The insects had a fresh weight of about 100 mg each and were separately collected in 25 ml tubes (Sarstedt, Verona, Italy), which were stored at −20°C until use.

Ten symptomless 2-year-old *P. pinea* cones were collected from the Natural Park of Migliarino – San Rossore and processed according to Luchi et al. (2005b). Briefly, each cone was surface-sterilized and split into two portions, one of which was used for DNA extraction and the other for isolation. After isolation, *D. pinea* was identified by the morphological features of the mycelium and conidia (Stanosz et al. 2001).

2.2 DNA extraction

Insects and cones (about 100 mg fresh weight per sample) were ground in liquid nitrogen. The WW from each sample was centrifuged for 3 min at 13 400 ***g***, and after removing the excess water the pellets were ground by micropestles in a 1.5 ml Eppendorf tube (Eppendorf, Italia, Milano, Italy).

DNA from all the samples was isolated with E.Z.N.A. Plant DNA kit (Omega Bio-Tek, Norcross, GA, USA), following manufacturer's instructions, and the concentration was measured using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.3 *Diplodia pinea* detection after real-time PCR

Diplodia pinea from all samples (insects, pine cones, WW and CS) was detected by real-time PCR using TaqMan™ chemistry as described in Luchi et al. (2005b). DNA samples were assayed in MicroAmpOptical 96-well plates (Applied Biosystems, Foster City, CA, USA), sealed with MicroAmp Clear Adhesive Film (Applied Biosystems). Real-time PCR was carried out using a 7300 Real-time PCR System (Applied Biosystems) following the protocol described in Luchi et al. (2005b). DNA of the *D. pinea* reference strain 3AP (Columbus, OH, USA) was used to calibrate the standard curve. Standard points, ranging from 25×10^3 pg DNA per tube to 0.32 pg per tube, were made using eight 1 : 5 serial dilutions of fungal DNA. Each point on the standard curve was assayed in triplicate.

The total DNA extracted from the insects had a much greater proportion of insect DNA than of *D. pinea* DNA. To exclude potential inhibitors and to test the sensitivity of the real-time PCR method on insects, fungal DNA was mixed with insect DNA. Each of the eight 1 : 5 serial dilutions, ranging from 25×10^3 pg DNA per tube to 0.32 pg per tube (isolate 3AP), was mixed with 80 ng of insect DNA per tube. The curve so obtained was compared with the standard curve where the fungal DNA was diluted in water.

To estimate the number of fungal conidia in artificially inoculated insects (CS and PCI groups), the amount of *D. pinea* DNA was correlated with the number of fungal conidia in a serial dilution. Serial dilutions of 1 : 1, 1 : 10, 1 : 100 and 1 : 1000 were prepared (1 : 1 corresponded to 4×10^4 conidia per ml). Each suspension (carried out in a volume of 1 ml) was used for DNA extraction (as described for the WW) and then quantified using real-time PCR.

2.4 Data analysis

The amount of *D. pinea* DNA in both insects and pine cones was expressed as pg fungal DNA/100 mg tissue. Differences in *D. pinea* DNA between treatment groups were detected by the analysis of variance (ANOVA) followed by Tukey's HSD *post hoc* test. The Pearson correlation was calculated between the amount of *D. pinea* DNA (pg/μl) from artificially inoculated insects and the number of propagules from the serial dilution conidia. The significance was evaluated at the 0.05 p-level. Statistical analysis was carried out using STATISTICA 6.0 (StatSoft Italia srl 2001).

3 Results

3.1 Standard curve

The standard curve exhibited a slope of −3.80, a square correlation coefficient (R^2) of 0.995 and a *y*-intercept of 38.1 (Fig. 1). The linear regression produced with eight dilutions of *D. pinea* DNA mixed with insect DNA had a slope of −3.68, a square correlation coefficient of 0.993 and a *y*-intercept of 37.2. This curve coincided with the standard curve obtained by the serial dilution of fungal DNA in water, and excluding the occurrence of any inhibitors in insect DNA (Fig. 1). The detection limit of the assay on insect DNA was 0.32 pg per tube (C_t = 39.27 ± 0.23), corresponding to 0.064 pg/μl of *D. pinea* DNA.

3.2 *Diplodia pinea* detection from insects and cones

Real-time PCR detected *D. pinea* DNA in all the insects tested. Significant differences in the amount of *D. pinea* detected were found between insect groups (F = 3.96; d.f. = 5; $p < 0.05$) (Fig. 2). The highest mean values of fungal DNA (1108.1 pg *D. pinea*/100 mg insect) occurred in insects inoculated with the conidial suspension (CS group). Lower but broadly similar amounts occurred in insects inoculated by crawling on infected cones (PCI group) and in unwashed insects from the forest of Vallombrosa (VF group) (Fig. 2). These DNA levels were similar to those detected in the symptomless pine cones, where they were confirmed by isolation on agar-media. The untreated, laboratory-grown control insects and the washed insects from the forest of Vallombrosa (VFW group) had the lowest amounts of *D. pinea* DNA, which were significantly different from those of the other groups (Fig. 2).

The WW from the insects contained *D. pinea* DNA in quantities ranging from 0.50 to 0.16 pg/μl.

A significant positive correlation (r = 1; $p < 0.05$) was found between the amount of fungal DNA and the number of conidia in the serial dilution. The correlation curve that quantified *D. pinea* conidia from mechanically inoculated insects exhibited a slope of 10.07, and a y-intercept of 14.81. Extrapolation data showed that the amount of fungal DNA extracted from insects inoculated with the CS corresponded to 71 ± 14.1 (mean ± SE) (CV%= 69) conidia per insect, and the amount of fungal DNA from insects inoculated by crawling on pine cones was 33 ± 16.6 (CV%=71) conidia per insect.

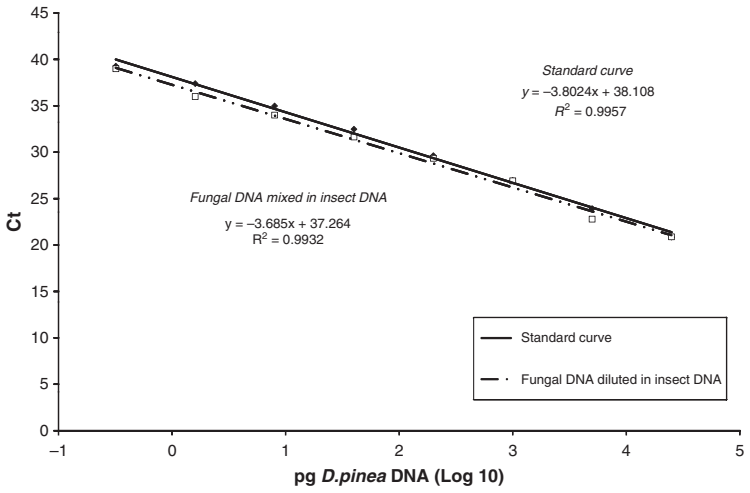

Fig. 1. Standard curve of *Diplodia pinea* generate by plotting the values of threshold cycle (C_t) in *y*-axis vs serial dilutions of fungal DNA (solid line). The dotted curve represent the same fungal serial dilutions mixed in insect DNA. The value of each standard point is the mean ± standard deviation of three replicates.

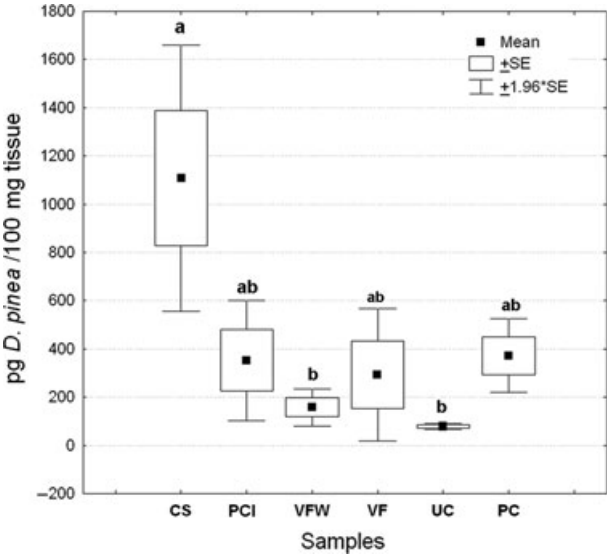

Fig. 2. Diplodia pinea DNA quantification on *Leptoglossus occidentalis* and pine cones. Insects from laboratory colony were artificially inoculated (CS, Conidial suspension; PCI, Pine cone inoculation) while those not treated were used as Uninoculated control (UC). Samples from forest included insects (VF, Vallombrosa forest; VFW, Vallombrosa forest washed) and pine cones (PC).

4 Discussion

Real-time PCR consistently detected *D. pinea* on the insects, even when these had been washed. This suggests that an association had indeed formed between the exotic *L. occidentalis* and the native *D. pinea*.

Over the last few years, invasive species have been one of the main causes of emerging infective diseases (EIDs) (Bandyopadhyay and Frederiksen 1999; Anderson et al. 2004). Outbreaks of EID occur either when an invading aggressive fungus finds an existing pathway to exploit, or when new vectors invade that are able to transmit a native pathogen more effectively (Bandyopadhyay and Frederiksen 1999). For example, Battisti et al. (1999) found that there was a long-standing association between the native seed bug *Orsillus maculatus* and the originally exotic fungus, *Seiridium cardinale*, and showed that the seed bug was now an effective vector of *S. cardinale* inoculum on cypress cones. In the United States, another close association has been found between the native beetle of elm bark, *Hylurgopinus rufipes*, and *Ophiostoma novo-ulmi*, the exotic agent causing Dutch elm disease (Millar et al. 1986). On the other hand, *De. valens*, a secondary bark beetle in North America, has recently been introduced to China where it has formed an association with native ophiostomatoid fungi, and particularly with *Le. procerum* (Lu et al. 2009).

Leptoglossus occidentalis and *D. pinea* have the same habitat, offering the same conditions for growth, and this explains why an association has arisen between them.

In the present work, this association or interaction was seen when real-time PCR detected *D. pinea* DNA occurred on both artificially inoculated *L. occidentalis* and *L. occidentalis* naturally infected in a forest. Experiments on laboratory-grown insects found that the conidia adhered to the body of the insect, as the highest amount of fungal DNA was detected after conidial inoculation.

Comparable amounts of *D. pinea* DNA were found with other inoculation procedures, as by insects crawling over infected pine cones, or when insects were collected from the forest, where they were probably naturally infected with conidia by coming in contact with infected trees.

As *D. pinea* inoculum can persist for a long time as a saprophyte on the parts of dead trees, such as cones and different coarse woody debris (Santini et al. 2008), the insects may also become infected with *D. pinea* by crawling on the tree litter.

Diplodia pinea was even detected on uninoculated laboratory-grown (control) insects that had only been fed on apparently healthy Austrian pine seedlings and shoots. The explanation for this is probably that the trees that were fed to these insects were already colonized by the fungus in latent form. The fact that nevertheless small amounts of *D. pinea* DNA were detected in these samples even after they had been washed could be due to the not quite perfect nature of the washing process, which left some conidia adhering to the insect; although it is also possible that when *L. occidentalis* sucked the young symptomless cones, it ingested some fungal hyphae or fungal DNA fragments occurring on the cones.

Although beetles are thought to be the main vectors of plant pathogens in forests, this is the first time a bug was found to act as a possible fungal vector in a forest. However insects, mainly the Heteroptera, have a vital role as vectors of various disease agents such as viruses, bacteria, phytoplasmas and fungi, on cotton, pistachio, citrus and soybean (Mitchell 2004). Heteroptera transmit pathogens mainly by their piercing–sucking mouthpart that they use in feeding (Mitchell 2004). Citrus and pistachio trees are infected with two yeasts, *Nematospora coryli* and *Ashbya gossypii*. Infection occurs just at the time when these trees are being fed on by the leaf-footed bug *Leptoglossus gonagra* F. The bug is clearly associated with these two yeasts, which have also been found in the digestive tract of the insect (Mitchell 2004).

The existence of an interaction between insects and fungi is essential for the spread of the fungi, and consequently for disease outbreaks. A fungus-insect association usually benefits both parties. In the *Orsillus maculates/S. cardinale* interaction, the insect benefits because the fungus opens the cones, enabling the nymphs to feed on the seeds, and the fungus benefits because the insect disseminates its conidia (Battisti et al. 1999). In the association between *L. occidentalis* and *D. pinea*, the fungus benefits by being spread by the insect to other pine trees, and to trees of different species that are hosts to both itself and the insect, and the insect benefits because the fungus could stimulate the tree to produce monoterpenes, which are the main attractants of the insect. Such a mutual benefit has also been shown to occur in American elms in the association between *O. novo-ulmi* and the beetle *H. rufipes,* in which the fungus changes the elm's semiochemical blend so that it attracts the beetle, and the beetle kills the elm and carries the fungus propagules to other host trees (McLeod et al. 2005). Similarly, a recent study on Chinese pine (*Pinus tabuliformis*) found that the fungus *Le. procerum* raised levels of 3-carene, a monoterpene attracting the insect *De. valens* (Lu et al. 2010) which is a vector of the fungus.

Climate change and increasing global trade links are extending the range of many insects, introducing them to areas where they did not occur before (Walther et al. 2009). If this is what has happened to *L. occidentalis,* its interaction with *D. pinea* can be expected to produce serious damage to *P. pinea*. The insect may also spread the fungus to other conifers, further increasing the spread of *D. pinea* and strengthening the synergy between *L. occidentalis* and *D. pinea* in Mediterranean forests.

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