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Sources of *Diplodia pinea* endophytic infections in *Pinus patula* and *P. radiata* seedlings in South Africa

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Summary

Diplodia pinea, an opportunistic and latent pathogen, can significantly affect *Pinus* productivity worldwide. Despite being studied in South Africa for almost 100 years, the source of *D. pinea* inoculum responsible for seedling infection is unknown. The aim of this study was to determine the role of seed in vertical transmission of *D. pinea* and to investigate sources of inoculum leading to horizontal transmission to pine seedlings. Surface-disinfected seeds were inoculated with spore and mycelium suspensions of *D. pinea* to determine its effect on germination. In addition, isolation of the fungus was performed from surface-disinfected seeds, asymptomatic seedlings collected from nurseries, plantations where pines naturally regenerate and recently established fields, to assess transmission and incidence of endophytic *D. pinea* infections. Inoculation of seeds with *D. pinea* spore suspensions affected speed and rate of germination. The fungus was isolated from surface-disinfected seeds in only a few instances (2–3%) and was not found in healthy seedlings collected from greenhouses and nurseries, suggesting that vertical transmission of the fungus does not occur or is rare. In contrast, *D. pinea* was isolated from 40% of seedlings obtained from the understory of mature *P. patula* trees showing that horizontal transmission from mature to young trees sustains the *D. pinea* inoculum in South African pine plantations.

1 Introduction

Diplodia pinea (=*Sphaeropsis sapinea*) is an endophyte that exists as a latent pathogens without visible symptoms on pines in many parts of the world. It can be isolated from healthy tissue of pine trees at all stages from seedling to maturity, mostly in branches, twigs and reproductive organs. The fungus can also be isolated from asymptomatic wood of mature trees (Bihon et al. 2010).

Diplodia pinea results in disease when trees are exposed to physiological stress (Swart and Wingfield 1991; Blodgett et al. 1997; Stanosz et al. 2001). Infection results in a variety of symptoms, including damping-off, stem cankers, tip dieback, blue stain and where severe, large-scale tree death (Palmer et al. 1987; Swart and Wingfield 1991; Stanosz et al. 2005). In South Africa, damage to *Pinus radiata* because of *D. pinea* was estimated to be up to 28% loss of volume and 55% potential loss of production after hail damage (Zwolinski et al. 1990). For this reason, *P. radiata* is not widely planted in South Africa in regions that receive summer rainfall (Swart and Wingfield 1991). However, *D. pinea* still remains one of the most important pathogens of *Pinus* spp. elsewhere in the country and particularly on *P. patula* after hail damage (Swart and Wingfield 1991).

Transmission of endophytic fungi can be horizontal through spores or mycelium, vertical (systemic) via seed infection or through a combination of these mechanisms (Carroll 1988). Many grass endophytes are seed-borne and persist within plants for the duration of the host life cycle (Carroll 1988; Schardl 2001). In this situation, seeds harbour viable propagules of the fungi that colonize the developing germlings, guaranteeing vertical transmission to the next generation (Schardl 2001; Ernst et al. 2003). Unlike grasses, most tree endophytes are thought to be transmitted horizontally (Arnold et al. 2003; Arnold 2007; Slippers and Wingfield 2007).

Diplodia pinea is commonly isolated from seedlings in nurseries (Palmer and Nicholls 1985; Stanosz et al. 2005, 2007) and from different parts of pine species including, needles, cones, branches, seed scales, seeds and piths of cones and mature wood (Vujanovic et al. 2000; Flowers et al. 2001, 2003; Smith et al. 2002). The sources of infection in USA nurseries were mainly infected pine trees used as windbreaks and leftover pruned branches (Palmer and Nicholls 1985; Stanosz et al. 2007) because the fungus can develop on dead pine tissue and litters of other hosts (Swart and Wingfield 1987). Conidia of *D. pinea* are released from pycnidia in the presence of moisture and disseminated by wind and rain splash. Dispersal of conidia was positively correlated with moist weather in South African pine plantations (Swart and Wingfield 1987). The fact that *D. pinea* was isolated from seeds and also found throughout the living tissue of seedlings, trees and cones has led to the assumption that the 'fungus can be transmitted via seed', and seeds were considered as the major sources of the inoculum in South Africa (Burgess and Wingfield 2002). This assumption has not been tested experimentally.

The objectives of this study were to investigate the transmission, presence and prevalence of *D. pinea* in seeds, seedlings from nurseries and the field, and to determine how seedlings become infected with the fungus. Pine seedlings were collected from different sources and isolation of the fungus conducted in the laboratory. Surface-disinfected seeds of *P. radiata* and *P. patula* were also inoculated with spore and mycelium suspensions to see its effect on germination and persistence in seedlings in the greenhouse.

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2 Materials and methods

2.1 Isolation from seeds

Seeds of *P. radiata* and *P. patula* were obtained from Karatara and Ngodwana nurseries, South Africa, respectively. Seeds were surface disinfested in 70% EtOH for one minute and 3.5% NaOCl for 2 min followed by 70% EtOH for 1 min. These treated seeds were then repeatedly washed using distilled water until clean water flowed out of the sieve. The seeds were then blotted on autoclaved filter paper and dried under room temperature. One hundred seeds from each species were randomly taken and plated in five Petri dishes (i.e. 20 seeds per Petri dish) containing 1% water agar (WA) m/v and incubated at 25°C in the dark. This was repeated for an additional 200 *P. radiata* and *P. patula* seeds at two different times to have representative information.

Growth of *D. pinea* was monitored daily, starting 2 days after incubation. Colonies appearing to represent *D. pinea* were transferred to fresh malt extract agar. Their identities were confirmed based on their cultural characteristics and internal transcribed spacer (ITS) rDNA sequences comparison (De Wet et al. 2003).

2.2 Seed inoculation and germination

Pinus radiata seeds were inoculated with mycelium and spore suspensions of *D. pinea*, strain CMW29144, to assess its effect on germination. Surface-disinfested seeds were dipped into a suspension made from a 10-day-old culture on 2% liquid malt extract (2% m/v Biolab malt extract) and 100 seeds were immediately plated on 1% water agar (WA) (1% m/v Biolab agar). Another set of *P. radiata* seeds was dipped in spore suspensions collected by vortexing pycnidia in Eppendorf tubes using sterile distilled water. One hundred of these seeds were plated on 1% WA the next day after treatment, and 100 seeds were plated after 20 days of storage at room temperature. Equal numbers of surface disinfested non-inoculated seeds were used as a control treatment. Analysis of variance was conducted and treatment effects (control; inoculation with mycelium suspension; inoculation with spore suspension and planting one or 20 days after inoculation) were tested at 5% probability level. Mean separation was evaluated using Student's t-test ($p = 0.05$) in JMP-5 (SAS Institute Inc., Carry, NC, USA).

Treated seeds were allowed to germinate and germination success was recorded from day 5. A seed was considered germinated when the radical protruded through the seed coat. Germlings were observed on the radical and plumule of the seedlings for defects because of the fungus. Germinating *P. radiata* seedlings, 10 spores treated and 10 non-treated controls were transferred to pots containing peat moss soil and placed in a greenhouse. These plants were allowed to establish for 2 months before they are harvested for *D. pinea* isolation.

2.3 Isolation of *Diplodia pinea* from seedlings

Isolations of *D. pinea* were performed from seedlings raised from (1) commercial nurseries, (2) naturally regenerated seedlings within a plantation, (3) seeds and seedlings grown in the greenhouse and (4) planted seedlings in an open field.

One hundred asymptomatic *P. patula* seedlings were collected from each of three commercial nurseries in the Sabie area. In addition, approximately 65 naturally regenerated 2-year-old seedlings were collected alongside plantations of mature *P. patula.* A second set of approximately 1-year-old transplanted seedlings were collected from a cleared pine stand, but where there were no mature trees in the close proximity, close to Ngodwana, Mpumalanga. Plants were collected in separate plastic bags and taken to the laboratory for isolation.

To assess the presence of *D. pinea* in the seedlings raised under a controlled environment in the greenhouse, surface-sterilized *P. radiata* seeds were sown in a tray containing peat moss soil. When germinated, seedlings were transferred into 11 cm diameter polyethylene bags. One year after transplanting, 100 seedlings were cut at the soil surface and isolation of the fungus was undertaken on 2.0% malt extract agar (2% m/v Biolab malt extract, 1.5%m/v Biolab Agar).

2.4 Persistence of *Diplodia pinea* in seedlings

Persistence of *D. pinea* was examined by inoculating the terminal shoots of 1-year-old *P. radiata* seedlings with a 5 μl spore suspension of strain CMW29144 at a concentration of 5.0×10^5 spores per millilitre. The suspension was applied using a micropipette and without damaging the shoots. The same amount of distilled water was applied on five equal sized seedlings for the control. The inoculated seedlings were placed in a greenhouse, covered by polyethylene sheets for the first 2 days to maintain humidity required for spore germination, after which plants were watered regularly. Five months after inoculation, isolations were made from all inoculated and non-inoculated seedlings. Isolates recovered were characterized using microsatellite markers as described by Bihon et al. (2010) to confirm their identity with the strain used for inoculations.

3 Results

3.1 Isolation of *D. pinea* from seeds

Of a total of 300 *P. radiata* seeds tested at three different times (100 seeds at a time), *D. pinea* was isolated from seven seeds (2.3%) as confirmed from their culture morphology and ITS sequences. No *D. pinea* was isolated from the 300 *P. patula* seeds.

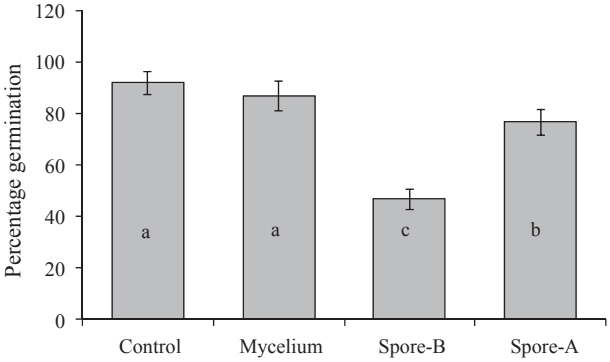

Fig. 1. Comparison of four treatments on the total percentage germination of *Pinus radiata* seeds. Treatments with different letters in the bar are significantly different at $P = 0.05$. Spore-A = Seeds treated with spore suspension and plated the following day, Spore-B = Seeds treated with spore suspension and plated after 20 days of storage.

3.2 Seed inoculation

Of the 100 surface-treated *P. radiata* seeds that were inoculated with a mycelial suspension of *D. pinea*, 87% germinated. This is in contrast to the 91% germination from the 100 seeds that were not inoculated. However, there was no significance difference between total germination percentage of mycelium suspension treated and control seeds at p = 0.05 (Fig. 1). There was some indication that seeds inoculated with a mycelial suspension germinated more slowly than the control seeds (Fig. 2). Thus, on the 7th day after plating on water agar, 43% of control seeds had germinated compared to only 19% of the treated seeds.

Treatment of seeds in the spore suspensions of *D. pinea* significantly reduced germination percentage (Fig. 3). In this experiment, 76% of 100 seeds that were inoculated and immediately plated had germinated, whereas only 53% of 100 seeds that had been inoculated and stored for 20 days germinated. The rest of these seeds never germinated. There were significant differences in total percentage germination between treatments at p = 0.05 (Fig. 1).

Lesions were observed on the radicals of some of the seedlings with both mycelium and spore treatments, while the non-treated controls showed no symptoms. No *D. pinea* could be isolated from ten seedlings germinated from the seedlings treated with a spore suspension after 2 months of growth in the greenhouse, which was also the case with seedlings from non-treated seeds.

3.3 Isolation from nursery and field-collected seedlings

No isolates of *D. pinea* were isolated from any of the 300 *P. patula* seedlings from three nurseries (100 seedlings each) randomly sampled in the Sabie area. *Diplodia pinea* could also not be isolated from the 100 1-year-old seedlings grown from seeds in a greenhouse and transplanted seedlings in the open fields. In contrast, the fungus was isolated from 26 of 65 (40%) naturally regenerated approximately 1- to 2-year-old seedlings collected alongside mature trees.

3.4 Persistence in inoculated plants

Diplodia pinea was recovered from 17 of 25 seedlings (68%) that were inoculated with a suspension of spores 5 months prior to termination of this experiment. The fungus was not isolated from control seedlings treated with distilled water.

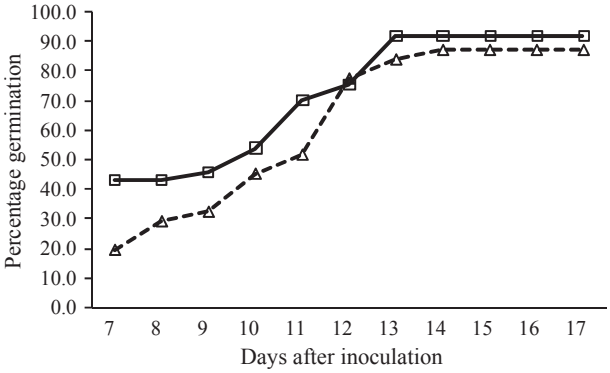

Fig. 2. Cumulative percentage germination of non-treated (solid line) and treated (broken line) *Pinus radiata* seeds treated in *Diplodia pinea* mycelial suspensions.

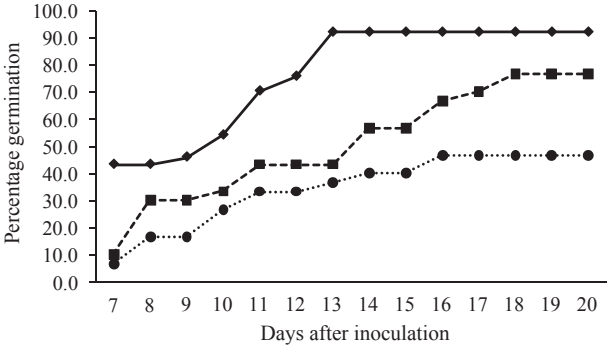

Fig. 3. Cumulative percentage germination of *Pinus radiata* seeds that are not treated (-♦- and solid line), and treated in *Diplodia pinea* spore suspensions, and immediately plated (-■-), or plated after 20 days of storage (-●-).

Microsatellite markers confirmed that the genotype of the recovered isolates was the same as that of the strain used in the inoculation (CMW29144).

4 Discussions

Results of this study confirmed that *D. pinea* is transmitted horizontally from mature trees, while no evidence could be found for any form of vertical transmission through seeds, as was thought previously. The fungus was isolated from surface-treated *P. radiata* seeds very infrequently, but not from *P. patula* seeds. It was not present in seedlings collected from nurseries or seedlings grown in the greenhouse, but was recovered from seedlings growing under mature trees. These results suggest that mature trees may be a major source of *D. pinea* inoculum in South Africa. An inoculation study also showed that *D. pinea* persists in inoculated *P. radiata* seedlings for at least 5 months after infection.

Diplodia pinea does not appear to frequently infect *Pinus* seeds. Previous studies reported isolating this fungus at a frequency of 4.6% from seeds of 12 *Pinus* species (Vujanovic et al. 2000). In another study, *D. pinea* was not obtained from healthy non-contaminated seeds, but was isolated from seeds collected from fallen cones on debris beneath trees (Fraedrich et al. 1994). The low rates of isolation in these previous studies and in the current study (2.3% on *P. radiata* and 0% on *P. patula*) suggest that seed transmission is not significant in the transfer of *D. pinea* but when large quantities of seed are transported, even this low percentage of infection could result in accidental introductions of this fungus.

The presence of the fungus in the seed is not necessarily an indication of transmission directly from the tree, as the seed could have acquired the fungus in the process of seed collection. High diversity of *D. pinea* genotypes in South Africa was previously reported (Burgess et al. 2004; Bihon et al. 2010) which could be because of multiple introductions, the presence of cryptic sex or mutation (Bihon et al. 2010). It was previously thought that imported seeds might account for the large diversity of *D. pinea* in South Africa (Burgess and Wingfield 2002). However, infected needles and cone tissues in seed batches could be the alternative sources of inoculum. It is also likely that seedlings or cuttings containing the fungus were imported into the country over the last three centuries and they could have contributed to introductions of the fungus.

Inoculation of seeds using a mycelial suspension of *D. pinea* affected germination speed, but the maximum percentage germination was not affected. Inoculation with a conidial suspension of *D. pinea* not only reduced the speed of germination but also significantly reduced percentage germination. These results are in agreement with previous studies. For example, *D. pinea* has been reported to cause mortality and reduce total germination of seeds of *Pinus oocarpa*, *Pinus caribaea* and *Pinus pseudostrobus* after inoculation with conidia (Rees and Webber 1988). Mortality of seeds because of inoculation with *D. pinea* can be attributed to the fact that the fungus could physically enter the seed coat via cracks or when the seed case opens prior to germination (Rees and Webber 1988; Fraedrich et al. 1994). Alternatively, the fungus may penetrate the testa physically or using enzymes (Rees and Webber 1988). Once the fungus is inside the seed, it can affect the germination process of the seed and ultimately kill the embryo. On the other hand, the effect of mycelial suspension on speed of germination could be because of physical interference of the mycelium itself on the plate through competition for resources, rather than pathogenicity of the fungus (Gure et al. 2005).

In this study, we show that *D. pinea* does not directly infect germinating seedlings. No infection could be detected in seedlings, even from seeds that were inoculated with conidia. Possible reasons for this could be that the seedlings had been raised from non-infected seeds or that *D. pinea* is present as small localized infections within a seedling, which makes it difficult to isolate (Flowers et al. 2006). More likely, however, is that spread and infestation of *D. pinea* is exclusively horizontally, similar to other tree endophytes (Arnold et al. 2003 & Arnold 2007, Ganley and Newcombe 2006). This was also shown in previous studies where only one type of fungal endophyte was isolated from 2% of *Pinus moniticola* seeds, but fungal endophytes were isolated from 57% of needles tested (Ganley and Newcombe 2006). In other seed-borne fungi (e.g. *Cladosporium cladosporoides*, *Epicoccum purpurascens*), no seedlings raised from inoculated seeds of white pine (*Pinus strobes*) and white spruce (*Picea glauca*) were infested after they were transferred to sterilized soil medium (Mittal and Wang 1993). The absence of the fungi in the seedlings raised from seeds therefore strengthens the previous reports that *D. pinea* infects seedlings when they are planted in the field, especially in close proximity to mature trees (Ganely et al. 2003).

Seedlings grown from various nurseries and open fields in South Africa were found to be free of *D. pinea*, in contrast to naturally regenerated seedlings collected alongside a mature pine plantation that had fairly high levels of infection. This was in contrast to the situation in USA where high seedling mortality was recorded in nurseries (Palmer and Nicholls 1985; Stanosz and Carlson 1996; Stanosz et al. 2007). In South Africa, seedlings are raised in trays on metallic beds that are closed with screens, whereas in USA, seedlings are often raised bare rooted under open conditions and surrounded by mature Pine tree windbreaks (Stanosz and Carlson 1996; Stanosz et al. 2005, 2007). Various authors have also shown that pruned branches from trees are sometimes left inside or around USA nurseries that contribute to inoculums levels in the nurseries (Stanosz and Carlson 1996; Stanosz et al. 2005, 2007).

Persistence of *D. pinea* as an endophyte in pine seedlings was confirmed by inoculating seedlings with an isolate of the fungus and recovering the same genotype five months later from healthy tissue. *Diplodia pinea* has previously been isolated from asymptomatic pine seedlings and mature trees, but the persistence of these infections has never been considered (Flowers et al. 2001, 2006; Smith et al. 2002; Stanosz et al. 2005). In a recent study, *D. pinea* has also been shown to be present in well-structured, healthy wood of *P. patula* trees (Bihon et al. 2010) and is most likely as the result of infection at different growth stages of a tree. *Diplodia pinea,* therefore, appears to be able to persist for very long periods of time in young and older tissues of a pine tree without causing disease.

In conclusion, it would appear that the South African nursery management conditions protect seedlings from infection by *D. pinea* and so reduce the transmission of the disease. Transmission of the pathogen is mainly horizontal through conidia that originate from mature pine trees. Planting disease-free seedlings in close proximity to older pines, or where there is debris from previous plantations, could contribute to their eventual infection. Removal of the diseased trees, twigs and debris from previous harvests is thus essential to inhibit conidial spread. Despite the fact that the fungus is present at a low level in seeds, it remains a potential route of introduction of new genotypes and thus movement of seed should be carefully monitored.

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