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The role of latent *Sphaeropsis sapinea* infections in post-hail associated die-back of *Pinus patula*

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Abstract

Sphaeropsis sapinea is an economically important pathogen of pines in South Africa. The most serious disease caused by this fungus is die-back of *Pinus patula* and *Pinus radiata* after hail. In this study, we investigate the role of latent *S. sapinea* infections in seed cones of *P. patula* in post-hail associated die-back. *P. patula* seed cones were found to be infected during the second year of development. Extensive colonization of all cone tissues occurred in the third year when cones mature, prior to seed discharge. Vegetative compatibility tests showed that *S. sapinea* is present as a single vegetatively compatible group in the third year seed cones. The presence of *S. sapinea* in colonized third year seed cones apparently result from a single successful infection in each cone. The probable role of latent infections by *S. sapinea* was considered in a case study of hail-damaged *P. patula* trees of three age classes. Mortality was most severe in a stand of 25-year-old trees (92%) followed by a stand of 14-year-old trees (32%) and a 5-year-old stand (9%). Isolations from diseased trees showed that *S. sapinea* could be recovered from darkly stained branch pith, which was continuous with infected pith in third year seed cones. In contrast, the fungus was absent in the non-discolored pith of branches on healthy trees of the same age. Our results indicate that *S. sapinea* infects branch pith tissue directly from previously infected, attached seed cones to cause rapid die-back and mortality of hail-damaged trees. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: *Sphaeropsis sapinea*; Pine; Die-back; Latent infections

1. Introduction

In South Africa, *Sphaeropsis sapinea* (Fr:Fr.) Dyko and Sutton was first reported in 1909 from the Fort Cunynghame State Forest in the eastern Cape Province (Lundquist, 1987a). Prior to 1930, *P. radiata* D. Don was the most commonly planted pine species and it was seriously damaged by *S. sapinea* (Lundquist, 1987b). Losses due to die-back caused by *S. sapinea* after hail damage was so serious that planting of *P. radiata* was discontinued in the summer rainfall and thus hail prone areas (Swart and Wingfield, 1991). By 1925, *P. patula* Schl. and Cham. had replaced most *P. radiata* in these areas and is now the most widely planted *Pinus* species in South Africa.

During the early 1930s it was believed that *P. patula* was tolerant to infection by *S. sapinea*. It was this fact that motivated its deployment in hail prone areas.

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Occurrences of die-back on *P. patula* occurred sporadically at first but steadily increased and by 1940, *P. patula* was no longer considered to be tolerant to invasion by the pathogen (Lundquist, 1987b).

By the early 1960s *S. sapinea* was considered to be the most important pathogen of plantation grown forest trees in South Africa (Lückhoff, 1964; Swart et al., 1985). The most serious losses associated with the fungus occurred after hail on *P. patula* in summer rainfall areas. It is estimated that between 1923 and 1983 there had been 11 outbreaks of *S. sapinea* induced die-back of *P. pinaster* and 25 of *P. radiata* in the southern Cape Province alone (Zwolinski et al., 1990). *S. sapinea* remains the most economically important pine pathogen in South Africa, despite the fact considerable effort is made to reduce its impact (Swart et al., 1985).

S. sapinea is well-known for its capacity to infect wounds (Wright and Marks, 1970; Palmer, 1991). These arise naturally or through forestry practices (Marks and Minko, 1969; Gilmour, 1964; Chou, 1984; Swart et al., 1985; Chou and MacKenzie, 1988) and insect damage (Swart et al., 1987). In South Africa, the pathogen is most frequently reported to infect pines through wounds caused by hail (Van der Westhuizen, 1968; Brown et al., 1981; Swart et al., 1987; Zwolinski et al., 1990).

S. sapinea has recently been shown to occur as a latent pathogen in various healthy pine tissues (Stanosz et al., 1997; Smith et al., 1996). Stanosz et al. (1997) reported that *S. sapinea* is able to cause latent infections in stem portions of nursery grown *Pinus resinosa* Ait. seedlings. Furthermore, Smith et al. (1996) showed that such latent infections are common in mature unopened seed cones of *P. patula* and *P. radiata* in South Africa. In the latter study, the fungus was recovered from seed cone pith tissue, seeds, seed wings and ovuliferous scales. The pith tissue of cones was found to be the most frequently infected. Both these authors speculated that this phenomenon might explain the rapid disease development under stress.

In this paper, we consider the probable role of latent infections caused by *S. sapinea* in *P. patula* seed cones, in post-hail associated die-back. Particular attention is given to the stage of cone development when infections occur and the number of infections that may occur in individual cones. The result of latent infections and the influence of tree age on mortality following hail damage is assessed using a case study. Finally, we consider the possibility that *S. sapinea* may be present in the form of latent infections in the bark of *P. patula* trees, and that these infections may contribute the development of stem and branch cankers associated with hail wounds.

2. Materials and methods

2.1. Laboratory studies

2.1.1. Stage of seed cone development

Collection of cones. Seed cones in three age classes, as well as current year pollen cones, were collected from a 14-year-old stand of *P. patula* in the Sabie area, Mpumalanga Province, South Africa. Seed cones represented current year, second year actively expanding and third year mature unopened cones. Thirty seed cones of each age class, as well as 30 pollen cones, were collected randomly in the stand (10 seed and pollen cones from each of three trees). All sampled cones were transported to the laboratory and stored at 4 °C. Isolations from these cones were done within 48 h of collection.

Isolation and culture methods. All cones were separated according to age, briefly submerged in 96% ethanol and flamed. In all cases, the cones were cut in half using sterilized pruning shears. Isolations from cone pith tissue, seeds, seed wings and ovuliferous scales were made by placing tissue pieces into Petri dishes containing 2% MEA (Malt Extract Agar, Biolab) supplemented with 200 mg/l chloramphenicol to suppress bacterial growth. From each third year cone, eight pieces of pith tissue, eight seeds, eight seed wings and eight pieces of ovuliferous scale were sampled. Seed wings were not sampled for the second and current year seed cones. The current year seed and pollen cones were cut in half and placed, with the cut surface facing the medium, without dissection. All plates were incubated at 23 °C for up to 2 weeks. Fast growing, darkly pigmented colonies were transferred to 2% MEA in culture vials and stored at 4 °C. These isolates were plated onto water agar, with sterile pine needles on the surface, to induce sporulation. Isolates were identified as *S. sapinea* based on pycnidial and conidial morphology.

2.1.2. Number of S. sapinea infections in cones

Selection of isolates. Ten, third year mature seed cones with high frequencies of *S. sapinea* recovery were selected for further study. Sixteen *S. sapinea* isolates were selected from each of these cones (four each from pith tissue, seeds, seed wings and ovuliferous scales) to represent a sampling of the entire cone.

Somatic compatibility tests. The 16 *S. sapinea* isolates originating from each of the 10 seed cones were evaluated for their diversity based on somatic compatibility groups (Anagnostakis, 1983). Isolates within each seed cone were paired in all possible combinations on oatmeal agar (OMA) plates. Representative somatic compatibility groups from each cone were further paired with isolates of all other cones.

Oatmeal (60 g/l) was steamed in a water bath at 70 °C for 2 h with periodic stirring. The solid particles were separated from the liquid by filtration through a double layer of cheesecloth resulting in approximately 600 ml of oatmeal broth. Agar (Biolab) (20 g) was melted in 400 ml distilled water added to the oatmeal broth and the final mixture was autoclaved. The medium was thoroughly mixed prior to dispensing into plastic assay dishes (25 cm square, Nunc). Isolates were placed approximately 1 cm apart on the OMA plates such that all isolates were paired with themselves and with all other isolates. Dishes were incubated at 20 °C in the dark for 4–6 days. Different somatic compatibility groups were identified based on the presence of barrage lines between incompatible isolates.

2.2. Field study

2.2.1. Behavior of latent S. sapinea infections following hail damage

Field site. During December 1995, a severe hailstorm occurred in the Sabie area, Mpumalanga Province, South Africa. The damage caused by the hail was widespread and uniformly damaged *P. patula* stands were in close proximity to each other. These stands included 5, 14 and 25-year-old trees. Mature seed cones were present in large numbers on the 14 (50–100 cones per tree) and 25-year-old (>100 cones per tree) trees, but were absent from the 5-year-old trees. In the months following the hailstorm up to March 1996, severe die-back and mortality occurred on trees. Undamaged 13 and 5-year-old stands of *P. patula* in the proximity were used as controls in isolation studies.

Isolations. During March 1996, diseased tissue including dead shoots as well as cankered branches and stems were collected from the three stands of *P. patula*. Isolations were made by placing small pieces of discolored cortex, pith and wood tissues from symptomatic branches and shoots onto the surface of 2% MEA, in Petri dishes. Isolates were identified as representing *S. sapinea* after sporulation.

Tissue colonization. In order to consider the role of latent infections, in seed cones, seven branches with attached cones were collected from hail-damaged 14-year-old *P. patula* trees. The branches and attached cones were split and isolations were made from the pith tissues of the seed cone, the stipe, the pith tissue continuous with the cone and the branch and the pith tissue of the branch. This isolation procedure was also used on seven branches and attached seed cones of undamaged 13-year-old *P. patula* trees that were collected outside the hail-damaged area. In addition, isolations were made from the pith tissue of seven branches of the damaged 5-year-old *P. patula* trees, that did not bear mature seed cones. Only 14-year-old trees were considered due to the fact that this was the only cone bearing age class with comparable undamaged trees available.

Assessment of mortality. Mortality varied greatly among the three age classes of *P. patula* trees damaged by the hail. The trees of all three age classes were in separate plantings in close proximity to one another and thus presented an opportunity to compare the outcome of die-back caused by *S. sapinea* after hail, directly amongst these different age classes. An estimation of the mortality in each age class of trees was done by scoring a block of 100 trees. Trees were scored only as dead or alive during September 1996.

2.2.2. Latent infections in the bark

Collection of bark samples. During March 1997, healthy intact bark was collected from 5-year-old *P. patula* trees damaged by hail as well as from trees of approximately the same age that were outside the hail area. Fifty bark disks were removed with a sterile cork borer from 10 healthy and 10 hail-damaged trees (five per tree). The five bark disk from each tree were sampled randomly from the main stem and branches. These bark disks were placed, with the inner bark facing the medium, on 2% MEA in Petri dishes. The fungal isolates growing from the bark pieces were identified as representing *S. sapinea* using the technique described earlier.

3. Results

3.1. Laboratory studies

3.1.1. Stage of seed cone development

S. sapinea was not recovered from the 40 current year seed or pollen cones sampled. The fungus was recovered from only 20% of the rapidly expanding second year seed cones and from 60% of the mature unopened third year seed cones (Table 1). The presence of *S. sapinea* in second year seed cones was restricted to the outer ovuliferous scales. In contrast, extensive colonization of the entire cone including the seeds, seed blades and cone pith tissue, of the third year seed cones was recorded (Table 1).

3.1.2. Number of *S. sapinea* infections in cones

S. sapinea isolates grew well on OMA and colonies made contact with each other within 2–4 days. Incompatible reactions resulted in dark mycelial barrage lines and were most obvious after approximately 5 days of incubation. After this time mycelial growth became very dense and the reactions were no longer clear. Compatible isolates merged without the formation of any barrage zones. *S. sapinea* was found, without exception, to be present in all 10 sampled seed cones as a single somatic compatibility group. Isolates from each of the 10 seed cones, however, accounted for a different somatic compatibility group.

3.2. Field study

3.2.1. Behavior of latent *S. sapinea* infections following hail damage

Isolation of the pathogen. *S. sapinea* was consistently isolated from diseased and dead shoots as well as cankers on branches and stems, representing all three *P. patula* age classes. The fungus was consistently associated with die-back symptoms and tree mortality.

Tissue colonization. The pith tissue of seed cones, cone stipes and branches was darkly discolored in the case of the 14-year-old *P. patula* trees damaged by hail (Fig. 1a). *S. sapinea* was consistently isolated from discolored pith tissues of the seed cones, the cone stipes, the pith tissues connecting the cones with the branches and the pith tissue of the branches. In contrast, discoloration of the branch pith in healthy undamaged 13-year-old trees that were outside the hail-damaged area was absent (Fig. 1b). In this case, *S. sapinea* was present only as latent infections in the seed cone and cone stipe pith tissues. Branches sampled from the hail-damaged 5-year-old *P. patula* trees did show canker development caused by *S. sapinea* surrounding hail wounds (Fig. 1c). The pith of these branches was, however, healthy, not discolored (Fig. 1d) and yielded no isolates of the pathogen.

Assessment of mortality. Mortality of hail-damaged *P. patula* trees varied greatly among different age classes. The 25-year-old stand had the highest percentage of dead trees (92%), followed by the 14-year-old stand (34%) and the 5-year-old stand (9%). We believe that the trees scored in each age class were representative of the whole stand and although the sample number was quite small, the differences was very clear.

Table 1
Presence of *S. sapinea* in different tissues of three age classes of *P. patula* seed cones and current year pollen cones

Cone age	% Cones infected	% Cone tissue infected with *S. sapinea*[a]			
		Seed	Seed blades	Pith tissue	Ovuliferous scales
Current year (pollen)	0	–	–	–	–
Current year (seed)	0	0	0	0	0
Second year (seed)	20	0	0	0	2.5
Third year (seed)	60	23	22	58	19

[a] 30 cones, 240 seeds (8 per cone), 240 seed blades (8 per cone), 240 pith tissues (8 per cone) and 240 ovuliferous scales (8 per cone) were sampled.

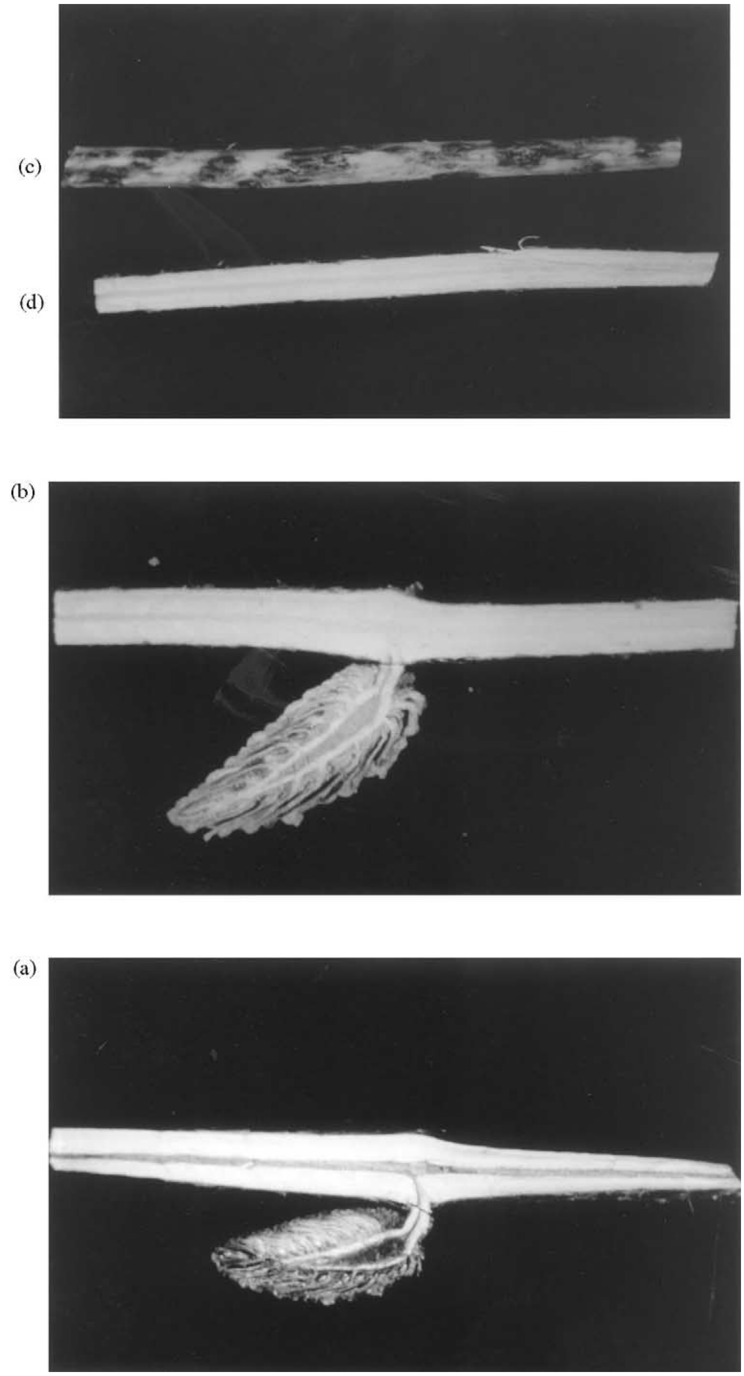

Fig. 1. (a) Darkly discolored branch and seed cone pith from a hail-damaged 14-year-old *P. patula* tree. (b) Absence of branch pith discoloration from a healthy 13-year-old *P. patula* tree. (c) Canker caused by *S. sapinea*, surrounding a hail wound. (d) Healthy non-discolored branch pith from a hail-damaged 5-year-old *P. patula* tree.

3.2.2. Latent infections in the bark

The bark disks sampled from both hail-damaged and undamaged 5-year-old *P. patula* trees were rarely infected with *S. sapinea*. From the 100 bark disks sampled, only 16 were infected with the fungus, nine from hail-damaged trees and seven from undamaged trees. Hail-damaged trees were thus not more heavily infected than undamaged trees.

4. Discussion

Results of this study have provided strong evidence that latent infections in mature *P. patula* seed cones, caused by *S. sapinea*, play an important role in post-hail associated die-back, and more specifically in tree mortality. *S. sapinea* appears to infect seed cones during the second year of development when they are actively and rapidly expanding. Our results support those by Peterson (1977), who also found that seed cones tend to be infected early in the growing season during the second year, when cones expand rapidly. Infections during this time are localized in the outer ovuliferous scales. Extensive colonization occurs only during the third year, prior to seed discharge, when cones become mature and inactive.

The fact that *S. sapinea* could not be recovered from current year seed and pollen cones suggests that these organs are not infected in the first year of growth. The reason for this situation is unclear. The sampling date may have had an influence on the time period that the current year cones were on trees, coupled with environmental conditions conducive to conidial discharge and successful infection. Thus, conidia of *S. sapinea* may have been present on the cone surface and destroyed with the flaming procedures followed, prior to isolation. Pollen cones may not be infected because they are short-lived. Second year seed cones are actively expanding and metabolically active, which possibly prevents *S. sapinea* from colonizing the cones extensively. The active defense mechanisms in such actively growing tissue would presumably inhibit infections, so that they remain discrete and localized.

The presence of *S. sapinea* in mature cones as a single somatic compatibility group per cone has three possible explanations. It could be argued that seed cones of *P. patula* were infected repeatedly during active expansion, but that these infections have a low rate of successful establishment. Alternatively, it could be that a single seed cone is successfully infected by a number of conidia belonging to the same vegetative compatibility group or that a unique infection event by only one conidium per cone takes place. The 10 seed cones sampled for the somatic compatibility group studies were, however, distributed throughout a stand of trees and *S. sapinea* isolates from each seed cone belonged to a different somatic compatibility group. It was also recently found that although *S. sapinea* is an introduced fungus in South Africa, it has a broad genetic base with many somatic compatibility groups in the Sabie area population (Smith et al., 2000). We, therefore, believe that repeated infections with a low rate of success are responsible for the presence of latent infections caused by *S. sapinea* in seed cones.

The colonization of the third year seed cones by *S. sapinea* is restricted to the different cone parts and the pith tissue of the cone stipe. The branch pith tissue of healthy undamaged 13-year-old *P. patula* trees was found not to be infected or colonized by *S. sapinea*. The pith tissues of third year mature seed cones as well as the pith tissues of seed cone stipes are metabolically inactive and brown (Fig. 1b). In contrast, the pith tissue of healthy branches is metabolically active (Esau, 1953) and green. The latent infection and colonization by *S. sapinea*, of third year mature unopened seed cones of *P. patula*, is thus confined to the inactive pith tissue of the third year mature seed cones, when trees are healthy and not stressed.

The colonization of branch pith tissue by *S. sapinea* changes after trees are stressed by environmental factors such as hail. The discoloration of the branch pith of hail-damaged 14-year-old trees was found to be extensive. Hail invariably stressed the affected trees to such an extent that the latent *S. sapinea* infections inside the pith tissue of third year seed cones were activated to spread into the pith tissue of the branches. The fungus thus appeared to overcome the defense barriers in healthy branch pith tissues rapidly and extensively. At this point we can only speculate that environmental stress may have profound impact on the efficiency of defense mechanisms (Woodward, 1992). In the case of environmental stress such as severe hail, *S. sapinea* is apparently able to colonize branch and stem pith tissues both proximal and distal to the points of infection. Here, these points of infection are primarily previously infected third year seed cones.

Branch pith colonization by *S. sapinea* appears to occur rapidly (Haddow and Newman, 1942; Marks and Minko, 1969). This would be facilitated considerably by the fact that the fungus is already prevalent in the pith tissue of the seed cones and the seed cone stipes. The pith tissue is largely comprised of parenchyma cells that are virtually devoid of chloroplasts in mature tissue. In older branches the cells are mostly empty and may act as accumulation sites for crystals and tannins (Esau, 1953). The presence of large intracellular spaces in this tissue (Esau, 1953) may explain the rapid rate at which it becomes colonized by *S. sapinea*.

Observations from this study suggest strongly that tree susceptibility due to *S. sapinea* infections and colonization after hail, increases as trees age. We believe that the extremely high mortality seen in the 25-year-old stand of *P. patula* is the direct result of extensive branch pith colonization by *S. sapinea*, which originates from the numerous third year seed cones on these older trees. The mortality of *P. patula* trees appears to be related to the large number of third year seed cones on older trees. This is in contrast to lower levels of mortality on young trees that do not carry mature seed cones.

S. sapinea was consistently associated with die-back symptoms as well as with branch and stem cankers of three age classes of *P. patula* damaged by hail. This was also true for 5-year-old *P. patula* trees that showed extensive die-back, often with more than half of the main stem that had died. In younger trees the mortality was, however, very low. The symptoms observed during this study are consistent with those reported for crown wilt (Chou, 1984; Haddow and Newman, 1942; Palmer, 1991) as well as branch and stem cankers (Palmer, 1991) caused by *S. sapinea* wound infections. We believe that infections of hail wounds on young trees that do not carry mature seed cones leads to crown wilt, with tree parts above the infection site dying off, but that these infections rarely result in tree death.

Colonization of the pith by *S. sapinea* moves down from a point of infection and subsequent canker development (Chou, 1984, 1987). This observation was confirmed in our study. Isolations from the pith tissue of branch segments from hail-damaged 5-year-old *P. patula* trees, revealed that this tissue is not colonized by *S. sapinea*, while healthy. These branch segments sampled were from the living lower parts of branches that had died back. Hail wounds on the bark of these branch segments had developed into cankers infected with *S. sapinea*, but the pith tissue was unaffected at the time of sampling. Hail wounds on dead and living branch segments were, however, of the same age and may have been infected by *S. sapinea* simultaneously. The fact that resistance to *S. sapinea* infection and disease development increases with wood and bark age (Chou, 1987), lower on stems and branches, may explain why these trees did not die-back indefinitely. Die-back was halted at a specific stage and trees started to recover and produced new growth as early as August, during the spring of 1996.

Despite the fact that *S. sapinea* is known to infect hail wounds (Van der Westhuizen, 1968; Brown et al., 1981; Swart et al., 1987; Zwolinski et al., 1990), we have found that the fungus also persists in asymptomatic bark of *P. patula* trees. The accepted dogma that hail wounds are infected by conidia of *S. sapinea*, and that this leads to rapid tree death is in our view, an oversimplification of the actual course of events. Latent *S. sapinea* infections in the bark probably become active after the onset of environmental stress factors such as hail. This would enable the fungus to colonize the damaged tissue surrounding such wounds. *S. sapinea* was found to occasionally cause lesions around control inoculation wounds in artificial inoculation trials conducted on *P. radiata* and *P. patula* (F.W. Wolfaardt, Department of Microbiology and Biochemistry, University of the Free State, PO Box 339, Bloemfontein 9300, South Africa, personal communication). These lesions may have been the result of latent infections of *S. sapinea* present in the bark of these trees.

S. sapinea sporulates prolifically on cones, after they have opened and discharged seeds (Haddow and Newman, 1942; Slagg and Wright, 1943; Laing and Chi, 1980; Peterson, 1981; Chou, 1984; Johnson et al., 1985; James et al., 1991). The inoculum produced on these retained cones plays an important role in the survival of the fungus. The conidia produced on these cones may infect current year shoots (Johnson et al., 1985) and also the second year seed cones. Seed cones thus appear to play an important role in the persistence and survival of *S. sapinea* on pine trees. It is our view that breeding programs aimed at selection for female sterility may lead to a significant reduction in damage caused by *S. sapinea*.

Results of this study suggest strongly that post-hail associated die-back of *P. patula* may result from two distinct forms of infection. Extensive die-back and high levels of mortality found in older trees bearing large numbers of mature third year seed cones. Alternatively, low mortality and recovery of young trees is found where cones are absent or in low numbers.

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