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The occurrence of *Lasiodiplodia theobromae* on *Pinus elliottii* seeds in South Africa

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Summary

Seeds of *Pinus elliottii* from clonal seed orchards in South Africa were examined in order to determine the cause of black discolouration and reduced germination prevalent in many seedlots. Symptomatic seeds were found to contain internal fungi whereas asymptomatic seeds showed no signs of fungal colonisation. Isolations were made from immature seeds in one-year-old cones and from seeds in mature two-year-old cones. *Lasiodiplodia theobromae* was identified as the predominant fungus present on symptomatic seeds. It was also isolated in relatively high percentages from mature cones, even though it was not commonly isolated from one-year-old cones.

Introduction

In South Africa, clonal seed orchards of *Pinus elliottii* Engelm. consistently produce relatively large numbers of diseased seed characterised by black discolouration and reduced viability (Cilliers *et al.*, 1993). A similar problem on *P. elliottii* seed in the USA has been associated with *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl. (Fraedrich & Miller, 1989). Rees (1988) showed that between 8 and 11% of the seeds from a *P. caribaea* Morelet seedlot from Nicaragua were infected with *L. theobromae*, and that the fungus occurred internally.

L. theobromae is a widespread unspecialised rot pathogen with a wide host range (Punithalingam, 1979). It has also been listed as an important seed-borne pathogen and has been isolated from the seeds of numerous non-coniferous plants (Barros *et al.*, 1985; Maholay & Sohi, 1977; Maholay & Sohi, 1982; Pizzanatto *et al.*, 1984). The fungus can survive for 12 months in seeds of bottlegourd and seven months in squash seeds (Maholay and Sohi, 1982). Seeds of jute (*Corchorus capsularis* L.) infected with *L. theobromae*, have been found to be the source of disease in the field and diseased trees subsequently produce diseased seeds (Fakir & Islam, 1993). The association of *L. theobromae* with seeds of coniferous species, as well as its pathogenicity to these seeds, has been demonstrated conclusively (Miller & Bramlett, 1979; Anderson *et al.*, 1981; Carneiro, 1986; Rees, 1988; Watanabe, 1988).

The incidence of *L. theobromae* on *P. elliottii* seeds appears to be related to cone har-

vesting and seed extraction procedures (Fraedrich & Miller, 1989). The degree of cone maturation at the time of harvesting may therefore be critical for colonisation of *P. elliottii* seeds by *L. theobromae*. Miller and Bramlett (1979) found that both *L. theobromae* and *Fusarium moniliforme* Sheld. var. *subglutinans* Wr. & Rienk. could cause extensive damage to nearly mature cones of *P. elliottii*, especially if wounds were present at infection courts. The fungus thrives under moist conditions and temperatures above 25 °C and these conditions are inadvertently established during the collection and precuring of cones prior to seed extraction.

The primary objective of this study was to determine the extent of fungal colonisation in symptomatic and asymptomatic *P. elliottii* seeds obtained from a seed orchard in the Eastern Transvaal province of South Africa. A second objective was to evaluate the influence of cone harvesting and especially storage practices on the incidence of fungal contamination.

Materials and methods

Germination studies. During March/April 1993, ten two-year-old cones were collected from three 20 year-old trees of each of four *P. elliottii* clones (E28, E6, E54, and E15) in a seed orchard near Sabie in the Eastern Transvaal province. Cones were transported to the laboratory in paper bags and stored at 4 °C for 24 hours. Fifty white, asymptomatic and fifty black, symptomatic seeds were randomly extracted from the cones of each clone and surface-disinfected in 3.5% NaOCl (m/v) for 5 minutes. Seeds from each clone were subsequently induced to germinate in the dark on sterile moist filter paper in Petri dishes at 25 °C. Germination of seeds was recorded as positive as soon as the radicle appeared.

Appearance of seeds. A suspension of black 'debris' present on the surface of black *P. elliottii* seeds was made by shaking 10 seeds in 10 ml of distilled water. The resultant suspension was examined under the light microscope at 400× magnification. Both black and white seeds (some cut longitudinally with a scalpel and some uncut) were fixed in 3% glutaraldehyde diluted in a cacodylate buffer ($Ca_{3(2)}AsO_2(Na)$, 21.4 g/l, pH 7.2–7.4) for 2 hours, rinsed in the same buffer solution and submerged in 1% osmium, diluted in the same buffer, for 2 hours. Seeds were dehydrated in a graded acetone series after which they were critical point dried. The seeds were mounted on stubs, coated with gold palladium and both their surfaces and interiors examined with a JSM 6400 scanning electron microscope (SEM).

Associated microflora. Seeds were screened for the presence of contaminant fungi using three techniques. A pilot trial was conducted with 100 seeds incubated on moist filter paper as described above. After incubation at 25 °C for 7 days, seeds were examined under low magnification with a dissecting microscope. Hyphae that were visible on the seed surfaces were removed with a sterile dissecting needle and transferred to 1.2% (m/v) water agar (WA). To facilitate sporulation of transferred fungal colonies, sterile

pine needles were placed on the surface of the agar and plates were incubated at 25°C under near-ultraviolet light. This technique was discontinued due to the laborious and time consuming task of picking the hyphae off seeds.

Isolation from symptomatic and asymptomatic seeds was subsequently performed on 5% Oxoid malt extract agar (MEA). After surface-disinfection in 3.5% (m/v) NaOCl, seeds were washed three times in sterile water. Four seeds were placed on each 90 mm Petri dish containing MEA and incubated at 25°C. The first visible fungal colony was transferred to WA and induced to sporulate as described above. Of each batch (100 asymptomatic and 100 symptomatic seeds from each of five *P. elliottii* clones), 50 were cut longitudinally under aseptic conditions in order to determine the presence of internally-borne fungi.

From each of four clones (E54, E15, E6 and E3) 200 symptomatic and 200 asymptomatic seeds were sampled. Due to a shortage of seeds, only 100 symptomatic and 100 asymptomatic seeds of an additional clone E28 were screened. Seeds were surface-disinfected as described above and placed, unbroken, onto plates of MEA containing rose bengal (30 ug/ml) to prevent excessive growth of bacteria and yeasts, and to limit fungal colony growth. The seeds were left unbroken because previous isolations on MEA showed no quantitative or qualitative differences in the fungi isolated from broken and unbroken seeds.

Dilution plating of debris. One hundred symptomatic *P. elliottii* seeds were suspended together in 5 ml sterile distilled water and agitated for 5 minutes until the black surface debris was suspended in the water. This suspension was then diluted to 16%, 3%, 0.5% and 0.08% and 1 ml of each dilution was plated onto each of three plates containing 4% Oxoid potato dextrose agar (PDA) and incubated at 25°C for 48 hours. Ten randomly chosen fungal colonies from each plate were transferred to fresh PDA plates. Where necessary, colonies were transferred to WA overlaid with pine needles and induced to sporulate under near ultra violet light for identification purposes.

Isolations from cones. Ten one-year-old cones were collected from each of the *P. elliottii* clones E28, E54, E7, E42 and E45 during March/April 1993 and screened for the presence of *L. theobromae*. The cones were transported to the laboratory in paper bags and stored at 4°C for four days until the isolations could be made. Those cones with insect-damage or obvious mechanical damage were avoided. Cones were not surface-disinfected and were cut in half and ten immature but fertilised seeds, were removed (figure 1C). The seeds, which were not surface-disinfected, were placed on PDA plates and incubated for 24 hours. Developing fungal colonies were then transferred to WA plates overlaid with sterile pine needles and induced to sporulate under near-ultraviolet light before they were identified.

Two-year-old cones of five clones of *P. elliottii* i.e. E28, E54, E7, E42 and E45 were collected during March 1993 at the optimum stage for harvesting. Cones were dislodged by hand and were immediately placed in paper bags. One batch of approximately 200 cones from each clone was placed in each of three hessian bags per clone and

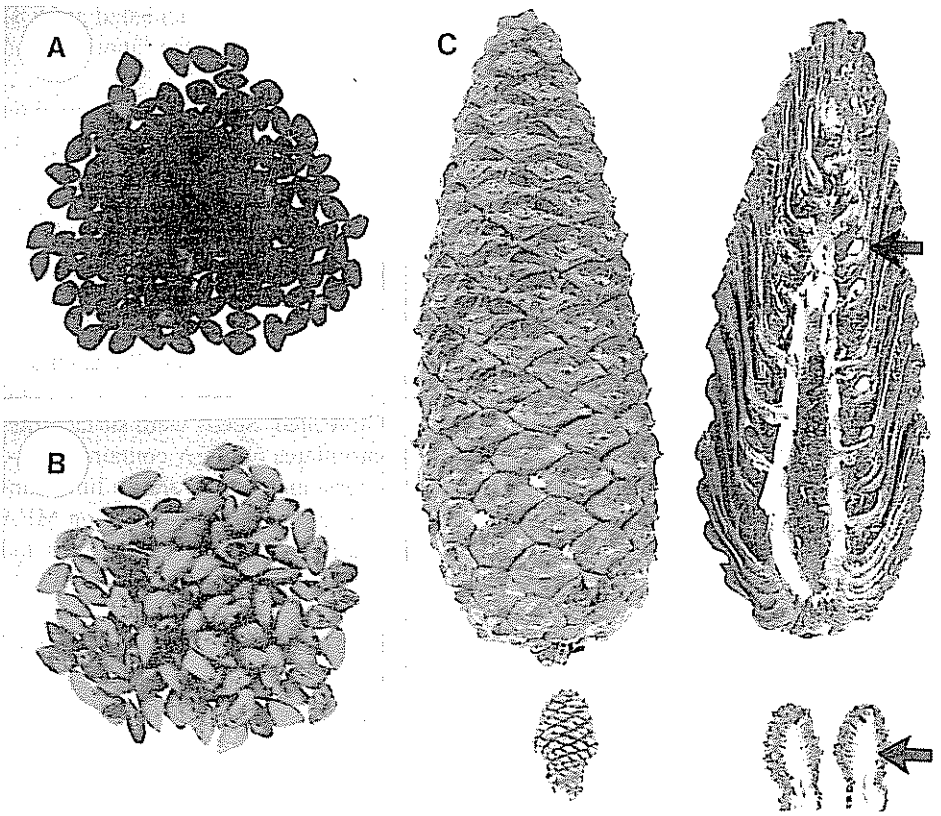

Figure 1. (A) Diseased (symptomatic) and (B) healthy (asymptomatic) seeds of *Pinus elliottii*, and (C) two- and one-year-old cones of *P. elliottii* with arrows indicating position of fertilised seeds.

closely stacked outdoors to prevent free movement of air between the bags. Bags were first wet thoroughly and then kept moist for the duration of the trial. A second batch of cones from each clone was spread on open kiln trays and kept under shelter to allow maximum air movement and drying. One hundred seeds from each clone were sampled immediately after harvest as described below. One bag containing mature cones from each clone was selected at two weekly intervals. At the same time, ten cones per clone were removed from the kiln trays and transported to the laboratory in paper bags. Seeds were extracted from case-hardened cones by sawing the cones longitudinally in half before removing the seeds (figure 1C). The blade of the saw was disinfected with 70% ethanol before each cone was cut open. Cones were not surface-disinfected before they were cut. Ten seeds, also not surface-disinfected, from each cone were placed onto 50 mm diameter Petri-dishes containing MEA (one seed per plate) and incubated at 25 °C for three days. Where necessary, fungal colonies were transferred to WA plates and identified as described above.

Results

Germination studies. Fungal mycelium grew profusely from symptomatic seeds during the germination studies, and these seeds failed to germinate. Asymptomatic seeds, which had no mycelium on their surfaces, showed germination percentages of 52, 86, 50 and 74% for clones E28, E6, E54 and E15, respectively.

Appearance of seeds. Conidia of various fungi excluding *L. theobromae* were observed by means of light microscopy in the suspension of the debris obtained from symptomatic seeds. Examination by means of SEM, revealed that superficial debris on symptomatic seeds contained numerous fungal hyphae but no conidia were clearly distinguishable (Figure 2A) in contrast to asymptomatic seeds which had no superficial debris (Figure 2B). Mycelium was also clearly visible inside symptomatic seeds (Figure 2C) but not within asymptomatic seeds (Figure 2D).

Associated microflora. Although saprophytic fungi, yeasts and bacteria were isolated on MEA and rose bengal, the dominant fungus isolated was *L. theobromae* (Tables 1

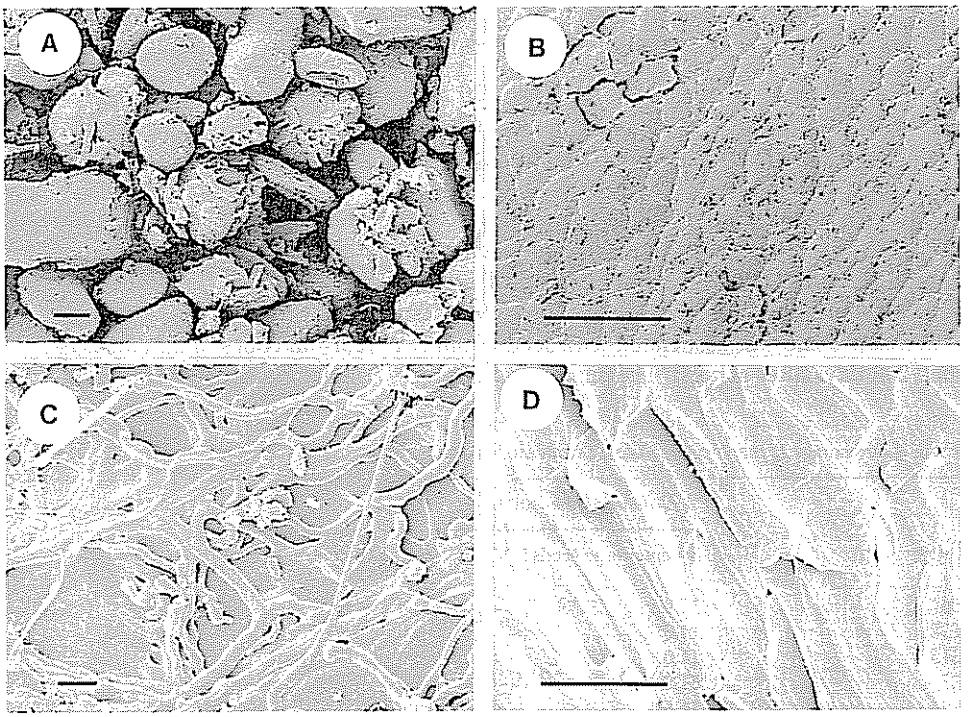

Figure 2. Scanning electron micrographs of *P. elliottii* seeds showing (A) superficial debris on the surface of diseased seeds, (B) clean surface of healthy seeds, (C) fungal hyphae inside diseased seeds, and (D) no signs of fungal colonization inside healthy seeds.

Table 1. Percentage *L. theobromae* isolated from *P. elliottii* seeds on malt extract agar (MEA).

	% *L. theobromae* isolated [a]			
	Symptomatic seeds		Asymptomatic seeds	
Clone	Uncut	Cut	Uncut	Cut
E6	48.0	52.0	36.0	20.0
E28	16.0	84.0	28.0	70.0
E15	2.0	34.0	6.0	4.0
E3	48.0	68.0	14.0	12.0
E54	82.0	88.0	0.0	10.0

[a] Two hundred seeds were screened per clone, 100 asymptomatic and 100 symptomatic seeds; 50 asymptomatic and 50 symptomatic seeds per clone were cut in half. The remaining 50 asymptomatic and symptomatic seeds were left uncut.

and 2). Other microflora were isolated in significantly lower percentages than *L. theobromae*. Other potentially pathogenic fungi such as *Fusarium oxysporum* Schlecht. emend. Snyd. & Hans. were isolated from both symptomatic and asymptomatic seeds in lower percentages (Table 3).

Dilution plating of debris. Only two of the 120 fungal colonies isolated from debris were identified as *L. theobromae*. Other fungal species that were commonly isolated were *Penicillium* spp., *Trichoderma* spp. and *Rhizopus* spp., especially at the lower dilutions.

Isolations from cones. Isolations from seeds extracted from one-year-old cones showed very low percentages of *L. theobromae* when compared to seeds obtained from mature cones (Table 4). The highest percentage of *L. theobromae* isolated from seeds of one-year-old cones was from clone E45 where 4% of isolations yielded *L. theobromae*.

L. theobromae was associated with seeds from two-year-old cones of all clones examined 2–3 days after harvest. The percentage of seeds, that had *L. theobromae* associated,

Table 2. Percentage *L. theobromae* isolated from seeds on MEA amended with 30 ppm rose bengal.

	% *L. theobromae* isolated [a]	
Clone	Symptomatic seeds	Asymptomatic seeds
E6	37.0	20.0
E28	14.0	6.0
E15	14.0	0.0
E3	61.0	14.0
E54	56.5	10.5

[a] Two hundred asymptomatic and two hundred symptomatic seeds were screened per clone except for clone E28 where 100 asymptomatic and 100 symptomatic seeds were screened.

Table 3. Microflora associated with symptomatic and asymptomatic *P. elliottii* seeds on MEA amended with 30 ppm rose bengal.

	% Occurrence	
	Symptomatic	Asymptomatic
Actinomyces sp.	0	0.82
Alternaria alternata	0	0.12
Aspergillus niger van Tieghem	1.65	6.00
Botryocrea sclerotioides (Hohn.) Petrak	0	2.47
Chaetomium fimicola Cooke	0	0.24
Cylindrocarpon didymum (Hartig) Wollenw.	0.12	0
Exserohilum rostratum (Drech.) Leonard & Suggs	0	0.12
Fusarium oxysporum Schl. emend. Snyd. & Hans.	0.35	0.59
Gliocladium roseum Bain	2.35	0
L. theobromae	36.58	11.75
Penicillium glabrum (Wehmer) Westling	5.76	5.18
Pestalotiopsis guepinii (Desm.) Stey.	0	0.12
Phialophora fustigiata-group	1.41	0.47
Sporothrix cf. state of *Ophiostoma stenoceras* (Robak) Melin & Nannfelt	0	0.24
Sterile mycelium	4.00	2.00
Trichoderma harzianum Rifai	0	0.24
Trichoderma sp.	1.88	2.12
Yeasts or bacteria	29.29	8.35
No microorganisms	16.61	59.15

varied from 19% in clone E42 to 97% in clone E45 (Table 4). Seeds screened from kiln trays after two weeks storage showed infection ranging from 38% in clone E42 to 90% in clone E7. Cones from the moistened hessian bags yielded higher percentages of *L. theobromae* than those stored on the trays after two weeks. Three of the five clones stored in bags showed a peak of *L. theobromae* after two weeks. After four weeks, three clones from cones stored on trays showed a peak of *L. theobromae*. After six weeks almost all the seeds from cones stored in bags and trays were colonised by various secondary fungi.

Discussion

The present study provides strong evidence that *L. theobromae* is associated with the phenomenon of black (symptomatic) seed from clonal seed orchards of *P. elliottii* in the Eastern Transvaal province of South Africa. This evidence is provided by the complete absence of fungal colonisation and the significantly greater germination percentage of white (asymptomatic) seed, in addition to the far greater percentage of *L. theobromae* isolated from symptomatic seed (Table 3). Our observations are therefore consistent with previous studies where *L. theobromae* was associated with diseased seeds of *P. caribaea* (Rees, 1988) and *P. elliottii* (Fraedrich & Miller, 1989).

Table 4. Occurrence of *L. theobromae* on seeds from one-yr-old cones of *P. elliottii* and from two-year-old cones stored in hessian bags and kiln trays for different periods of time.

	% *L. theobromae* isolated [a] Clone				
Cone treatment	E28	E54	E7	E42	E45
1-yr.-old cones	0	3	2	1	4
2-yr-old cones					
At harvest	55	46	51	19	97
14 Days after harvest					
Cones from bags	62	59	80	78	72
Cones from trays	49	56	90	38	52
28 Days after harvest					
Cones from bags	49	41	68	68	61
Cones from trays	72	74	71	40	88
42 Days after harvest					
Cones from bags	5	36	3	13	52
Cones from trays	9	25	13	LD[b]	35

[a] One hundred one-yr-old seeds were screened per clone. At harvest, 100 seeds were screened per clone and at each time interval after harvest 200 seeds were screened per clone, 100 from bags and trays respectively. All isolations were performed on MEA.

[b] LD = Lost data.

The mode of infection by *L. theobromae* of *P. elliottii* seeds is unclear. Since *L. theobromae* was isolated from immature, one-year-old cones in the present study, it appears that the fungus becomes established in a cone at a very early age. Infection could possibly occur as a result of mechanical or insect damage (Miller & Bramlett, 1979) or during fertilisation by means of internally infected pollen grains (Rees, 1988). The fungus then presumably colonises the cone tissue during the following year depending on suitable climatic conditions. When cones are harvested at two years, conditions for more extensive fungal colonisation can become more favourable depending on moisture and temperature conditions during storage. According to Kietzka (1992), seeds from *P. elliottii* cones stored at 10 °C and below displayed a significantly higher germination percentage than seeds from cones stored at higher temperatures presumably because this was less conducive to colonisation by *L. theobromae*.

In the present study, *P. elliottii* seeds from cones stored in bags and kiln trays both yielded relatively high percentages of *L. theobromae*, the only difference being in the number of weeks following harvest that the percentage *L. theobromae* isolated reached a peak (Table 4). The high percentages of *L. theobromae* isolated from cones stored in bags in the first three weeks of the trial, may possibly be attributed to the cones being colonised extensively in bags by *L. theobromae*, whereafter the decaying cones were colonised by secondary, saprophytic fungi. This is supported by the fact that after six weeks, both the

cones from the trays and those from the bags yielded seeds with very low percentages of *L. theobromae* due to a profusion of extraneous fungi which hampered isolation of *L. theobromae*. It would therefore appear that *L. theobromae* took longer to colonise cones and seeds stored in the open trays because of less favourable environmental conditions, and thus the onset of secondary colonisation by other fungi was delayed.

It is interesting to note that the common pine pathogen, *Sphaeropsis sapinea* (Fr.) Dyko & Sutton, which is closely related to *L. theobromae*, was not isolated from diseased *P. elliottii* seeds in the present study. There is, however, some consistency regarding this finding and the results of other workers who isolated *S. sapinea* from a very small percentage of diseased *P. elliottii* seeds produced in certain seed orchards in the southeastern USA (Anderson *et al.*, 1981; Anderson, Belcher and Miller, 1984; Fraedrich & Miller, 1989). However, in subsequent studies *S. sapinea* has been isolated from diseased *P. elliottii* seeds produced in other seed orchards in the USA (Fraedrich, *pers. comm.*). The association of *S. sapinea* with diseased *P. elliottii* seeds should therefore be the subject of further investigation.

The results of the present study suggest that environmental conditions prevailing from the time of fertilization to when cones are harvested, and during their subsequent storage, play a crucial role in determining infection by *L. theobromae* and the rate of cone and seed colonisation by the pathogen. A preliminary control strategy would therefore be to spray cones periodically with a suitable fungicide starting from the time of fertilisation to harvesting and then to store the harvested cones at 10°C prior to seed extraction. Masuka (1991) found Antiblue and Biocide to be effective against both *L. theobromae* and *S. sapinea* on sapwood chips. Runion and Bruck (1988) found that a thiabendazole-dimethyl sulfoxide soak increased the *in vitro* germination of *P. palustris* that was internally infected with *Fusarium subglutinans* (Wollenw. & Reink.) Nelson, Toussoun & Marasas. Further studies are, however, required to accurately determine the factors that influence infection and colonisation of *P. elliottii* cones by *L. theobromae* before final control measures can be prescribed.

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