Pythium irregulare Associated with *Pinus* Seedling Death on Previously Cultivated Lands

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ABSTRACT

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A serious root disease of *Pinus patula* seedlings has occurred in the northeastern Cape forestry region of South Africa during the past 3 yr. Mortality as high as 100% was experienced 4–5 mo after seedlings were planted on previously cultivated agricultural lands. No mortality, however, occurred in plantings on virgin lands. Where seedlings survived on previously cultivated lands, their growth compared poorly with those on virgin lands. *Pythium irregulare* was consistently isolated from diseased roots of *Pinus patula*, as well as from the soil of previously cultivated lands. *P. irregulare* was highly virulent when artificially inoculated onto 4-mo-old *Pinus patula* and 2-mo-old *Eucalyptus grandis* seedlings. *P. irregulare*, therefore, appears to be an important factor associated with deaths of *Pinus patula* on previously cultivated agricultural lands.

South Africa is a country characterized by poor timber resources. The forestry industry in the country, therefore, depends almost entirely on plantations of exotic *Pinus* spp., *Eucalyptus* spp., and *Acacia mearnsii* De Wild. Species of *Pinus* and *Eucalyptus* are most widely planted (90%), and they occur in approximately equal proportions (1). Seedlings of these trees are established in containerized nurseries and are planted out after approximately 4 and 6 mo for *Eucalyptus* and *Pinus* spp., respectively.

Recently, a serious disease problem has occurred in the northeastern Cape Province, where newly planted *Pinus patula* Schlechtend & Cham. seedlings failed to establish. The area involved was brought under forestry cultivation in 1989 and includes virgin grassland and 20,000 ha of previously cultivated agricultural land on which maize, wheat, and oats were grown. The disease of *Pinus patula* seedlings occurred only on the previously cultivated lands, and mortality was greater than 95% in most cases.

Pinus patula seedlings died approximately 4–5 mo after they were planted on previously cultivated lands. The first visible symptom was necrosis of needle terminals (Fig. 1A), after which seedlings rapidly wilted and died. Differences in pine stands on previously cultivated and virgin lands were evident on the Ronan farm in the Ugie district, northeastern Cape Province (Fig. 1B), where the previously cultivated land was replanted five times with *Pinus patula*. The growth of the seedlings that survived on previously cultivated lands compared poorly with the growth of those on virgin lands. The surviving seedlings were stunted, and root development was poor (Fig. 1C). Trees failed to become established on the sites despite five consecutive plantings.

The failure of forest trees to become established on previously cultivated lands has been reported in the United States (2,21,30). In these cases, the problem was recognized to be complex and not due to a single factor. However, various pathogens were associated with this establishment problem, including *Fusarium* spp., *F. subglutinans* (Wollenweb. & Reinking) P.E. Nelson, T.A. Toussoun, & Marasas in particular, and *Macrophomina phaseolina* (Tassi) Goidanich (21).

The failure of pines to become established on previously cultivated lands in South Africa has resulted in a considerable financial loss. Therefore, the objective of this study was to investigate the role of pathogens in the establishment of new plantations of *P. patula*.

MATERIALS AND METHODS

Site preparation. The mountainous nature of the Ugie district required the construction of ridges for planting to conserve soil and water. The practice includes the removal of a 50-cm strip of topsoil (20 cm deep) on both sides of a deep rip line. The soil was then heaped onto the rip line to construct the planting ridges. A 40-cm strip of soil was left between the areas where the topsoil was removed. Seedlings were then planted on top of the planting ridges.

Isolation and identification. Isolations were made from roots, as well as from soil in the rhizosphere of dying trees. Ten root and soil samples were selected arbitrarily from each of seven previously cultivated and seven virgin lands in the Ugie district. Each soil sample was thoroughly mixed and divided into two separate samples. Ten *Citrus* leaf disks were used to bait each sample (9). These were then transferred to a selective medium for the isolation of Oomycetes (33) and to a hymexazol medium for the isolation of *Phytophthora* spp. (32). Root segments from diseased seedlings were thoroughly washed in running tap water and plated on both selective media. Fungal isolates were transferred to cornmeal agar (CMA) for identification (35). The presence of the oomycetous fungi was quantified by taking the number of leaf disks yielding the pathogen(s) divided by the number of leaf disks used in baiting.

In addition to the survey above, 168 root and 168 soil samples of the same trees were arbitrarily taken from the Ronan land. *Pinus patula* seedlings on this site had a mortality rate of 99% for five consecutive plantings over the previous 3 yr. In general, the soil samples were taken on top of the planting ridges in the rhizosphere of the seedlings. However, 20 additional soil samples were taken from the area adjacent to the planting ridge where the topsoil layer was removed and from the areas where the soil was not removed (between planting ridges). Isolation and quantification of the oomycetous fungi was the same as in the first survey of previously cultivated and virgin lands.

The presence of fungal pathogens other than Oomycetes was investigated by incubating all diseased plant material in moist chambers. Transfers were made to potato-dextrose agar (PDA) for identification.

Damping-off tests. Inoculum of the two *Pythium* spp. most commonly isolated from soil of the Ronan previously cultivated land was increased in a sterile growth medium consisting of 200 ml of perlite, 60 g of cornmeal, and 70 ml of distilled water mixed in 500-ml Erlenmeyer flasks. The medium was autoclaved twice with a 24-hr interval between sterilization and shaken thoroughly after each sterilization. The medium was inoculated with PDA disks colonized

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with mycelium of the test fungi and incubated for 3 wk at 25 C in the dark.

Seeds of *Pinus patula* and *Eucalyptus grandis* A.W. Hill ex Maiden were surface disinfested for 1 min with 96% ethanol, rinsed twice with sterile distilled water, and allowed to germinate before pathogenicity tests were initiated. Pine bark composted for 7 mo, after which it was twice autoclaved at 121 C and 100 kPa with a 24-hr interval, was used as growth medium. One gram of inoculum was placed 1 cm below the germinated seed in each growth tube ($3 \times 3 \times 10$ cm^3). Medium inoculated with sterile PDA was used for control inoculations. Tests were conducted under greenhouse conditions with a temperature range of 23–26 C. Twenty seedlings of each species were used per treatment. Percent mortality was measured 3 wk after inoculation. Fungi were reisolated from diseased seedlings on culture dishes containing pimaricin (31).

Seedling pathogenicity tests. The pathogenicity of all the *Pythium* spp. isolated was tested on 4-mo-old *Pinus patula* and 2-mo-old *E. grandis* seedlings. Tests were conducted under greenhouse conditions with a temperature range of 15–32 C. Seedlings were cultivated in an unsterilized composted pine bark medium prior to inoculation. Preparation of inoculum was the same as for the damping-off tests. After incubation of the inoculum, the contents of five flasks were thoroughly mixed with 5.6 kg of steam-sterilized sand. The final ratio of inoculum to sand was approximately 1:8 w/w.

The bark medium was carefully removed from the roots, and seedlings were immediately planted in the sand–inoculum mixture. Bark was removed from seedling roots because of a possible inhibition effect on *Pythium* infection (10,14,15,28). Six seedlings were planted in each seedling tray. Four trays were used for each *Pythium* isolate on both *Pinus patula* and *E. grandis*. Seedling trays were arranged in a complete randomized design. Each seedling tray received 100 ml of water per day. Percent mortality was measured after 4 wk. Reisolations from inoculated and control seedlings were made on a selective medium (33).

Statistical analysis. The numerical data obtained throughout the study were subjected to an analysis of variance. Means were tested for significance according to Tukey's procedure (29).

RESULTS

Isolation and identification. Five species of *Pythium* from virgin lands and seven from previously cultivated lands were isolated from soil and root samples associated with *Pinus patula* (Table 1). *Pythium irregulare* Buisman was the most commonly occurring species associated with soil and diseased roots of *Pinus patula* from previously cultivated

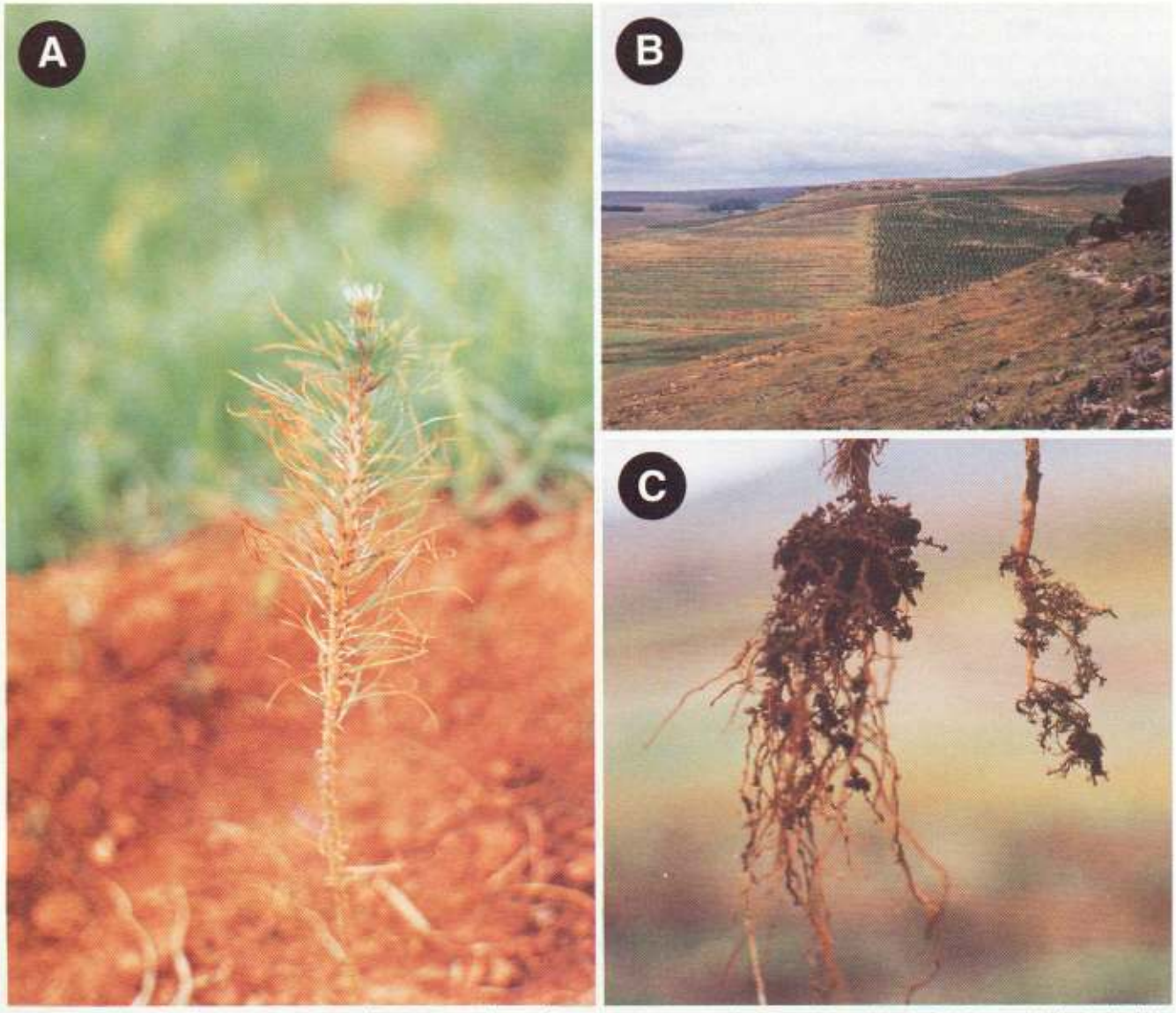

Fig. 1. (A) Necrosis of needle terminals of a *Pinus patula* seedling planted on a previously cultivated land. **(B)** Ronan virgin land (right) and previously cultivated land (left) after the previously cultivated land was replanted five times. **(C)** Roots of *Pinus patula* seedlings from a previously cultivated land (right) and from a virgin land (left).

lands. *Pythium* Group F, a heterothallic species with swollen, noninflated sporangia (35), was the second most common species isolated from soil and roots on these lands but the most commonly occurring species on virgin lands. Low populations of other *Pythium* spp. were associated with previously cultivated lands as well as virgin lands.

The most commonly occurring *Pythium* species associated with roots and surrounding soil of diseased *Pinus patula* seedlings from the previously cultivated Ronan land was *P. irregulare* (Table 2). *Pythium pyrilobum* Vaartaja and *Pythium* Group F were also associated with a few root and soil samples of *Pinus patula*. In contrast, *P. irregulare* was relatively uncommon (5%) in the associated virgin land.

P. irregulare was commonly isolated from soil collected on the planting ridges (99.2%) and between the ridges (32.4%). The lowest incidence of *P. irregulare* occurred in soil samples taken adjacent to the planting ridges (8.1%). However, the incidences of *P. irregulare* in soil from areas between the planting ridges were markedly lower than those from soil on the planting ridges and in the rhizosphere of diseased seedlings (Table 2).

Isolations from the aboveground parts of dead seedlings yielded *Sphaeropsis sapinea* (Fr.:Fr) Dyko & Sutton, *Fusarium graminearum* Schwabe, and a species of *Diaporthe*. None of these fungi was consistently isolated from the plants. They were, therefore, not considered to be an important component of the disease.

Damping-off tests. *P. irregulare* was highly virulent on postemergent *Pinus patula* seedlings, killing 76% of plants within 3 wk (Table 3). In contrast, *P. pyrilobum* was less virulent on this host, killing only 50% of the seedlings. Both fungi were also pathogenic on *E. grandis* seedlings, although virulence was much lower on this species (Table 3). *P. irregulare* was more virulent than *P. pyrilobum* on *E. grandis*. No seedlings in control inoculations displayed disease symptoms, and the pathogens were reisolated only from inoculated plants.

Seedling pathogenicity tests. *P. irregulare* was more virulent than *P. tardicrescens* Vanterpool, *P. spinosum* Sawada, and *P. acanthophoron* Sideris on *Pinus patula* (Table 4). There was 100% mortality on *E. grandis* seedlings inoculated with *P. irregulare*, *P. pyrilobum*, *P. tardicrescens*, and *Pythium* Group F. Other *Pythium* spp. displayed lower virulence on this host (Table 4). Symptoms associated with infection by *P. irregulare*, *P. acanthophoron*, and *P. pyrilobum* on 4-mo-old *Pinus patula* seedlings were the same as those observed under natural conditions.

In the controls, 8.4 and 4.2% of the *Pinus patula* and *E. grandis* seedlings, respectively, died. This was apparently due to replant shock. No pathogens were isolated from these dead control plants, but the appropriate *Pythium* sp. was reisolated from all dying seedlings that had been inoculated.

DISCUSSION

The results of this study have shown that a number of *Pythium* spp. are associated with establishment deaths of *Pinus patula* on the previously cultivated agricultural lands under consideration. Of these, only *P. irregulare* was consistently isolated both from dying plants and from soil. Among the pathogens, this species is, therefore, considered as the most important component of the establishment failures of *Pinus patula* in the region investigated. These results also support the view of Mitchell et al (21) that pathogens can be an important component of the deaths of *Pinus patula* during establishment on previously cultivated lands.

A high population of *Pythium* spp., particularly *P. irregulare*, occurred on previously cultivated lands as compared with lands not previously cultivated. Deaths during establishment of pines have previously been recorded under similar situations elsewhere (21,30); and the situation is, therefore, not unique. It is assumed that cultivation of maize, wheat, and oats has led to a buildup of populations of *Pythium* spp. (12,25,37). *P. irregulare* is the most virulent of the *Pythium* spp. to those hosts in South Africa (25).

Many *Pythium* spp. were isolated in this study, and various of these were

Table 1. Percent leaf disks employed in soil baiting and roots of *Pinus patula* seedlings from previously cultivated as well as virgin lands from which *Pythium* spp. were isolated

	Virgin lands		Cultivated lands	
***Pythium* species**	**Soil (%)[y]**	**Roots (%)[z]**	**Soil (%)[y]**	**Roots (%)[z]**
P. acanthophoron	12.1	2.1	8.4	1.5
P. irregulare	17.5	5.6	94.2	82.6
P. pyrilobum	0.0	0.0	5.7	3.7
P. spinosum	5.3	0.0	17.8	9.9
P. tardicrescens	21.3	9.7	11.1	3.2
Pythium Group F	41.2	25.5	33.3	22.0
Pythium sp.	0.0	0.0	2.5	0.0

[y] Each value represents the percent leaf disk yielding *Pythium* spp. of 70 soil samples from seven formerly cultivated or seven virgin lands.
[z] Each value is the percent roots of 70 *Pinus patula* seedlings from seven formerly cultivated or seven virgin lands from which *Pythium* spp. were isolated.

Table 2. Percent isolation of *Pythium* spp. from roots as well as soil in the root zone of diseased *Pinus patula* seedlings from the Ronan formerly cultivated and virgin land

	Isolation (%)[x]			
	Virgin land		Cultivated land	
***Pythium* species**	**Soil**	**Roots**	**Soil**	**Roots**
P. irregulare	5.0	0.0	99.2	87.5
P. pyrilobum	0.0	0.0	35.3	17.6
Pythium Group F	31.0	19.6	23.7	16.8

[x] Each value represents the average percent isolation of the *Pythium* spp. from 168 root and 168 soil samples.

Table 3. Postemergence damping-off of *Pinus patula* and *Eucalyptus grandis* seedlings inoculated with *Pythium irregulare* and *P. pyrilobum*

	Mortality (%)[y]	
***Pythium* species**	***P. patula***	***E. grandis***
Control	0 a[z]	0 a
P. irregulare	76 c	38 c
P. pyrilobum	50 b	18 b

[y] Each value is the average of 20 germinated seedlings 3 wk after inoculation with the respective *Pythium* spp.
[z] Values in each column followed by the same letter are not significantly different ($P \leq 0.01$) according to Tukey's procedure for comparison of means. *Pinus patula* CV = 10.5%. *E. grandis* CV = 14.0%.

Table 4. Mortality of 4-mo-old *Pinus patula* and 2-mo-old *Eucalyptus grandis* seedlings inoculated with *Pythium* spp. isolated from previously cultivated lands

	Mortality (%)[y]	
***Pythium* species**	***P. patula***	***E. grandis***
P. acanthophoron	37.5 cd[z]	8.4 a
P. irregulare	83.3 e	100.0 b
P. pyrilobum	29.1 bcd	100.0 b
P. spinosum	48.5 cd	0.0 a
P. tardicrescens	50.0 d	100.0 b
Pythium Group F	0.0 a	100.0 b
Pythium sp.	25.0 bc	8.4 a
Control	8.4 ab	4.2 a

[y] Each value is the means of 24 seedlings.
[z] Values in each column followed by different letters differ significantly ($P \leq 0.01$) according to Tukey's procedure for comparison of means. *Pinus patula* CV = 13.0%. *E. grandis* CV = 9.0%.

shown to be pathogenic to seedlings of *E. grandis* and *Pinus patula*. The results of this study confirm those of Vaartaja (34), who showed that *P. irregulare* and *P. pyrilobum* can cause damping-off of pine and *Eucalyptus* seedlings. This is the first report of *P. pyrilobum* from South Africa. Various other *Pythium* spp. can also act as pathogens of *Pinus* and *Eucalyptus* spp. (20,26,34,36). These fungi also played a role in mortality of young seedlings during establishment in the field. However, because *P. irregulare* was most frequently isolated, it is believed to be the primary cause of the problem.

P. irregulare, *P. tardicrescens*, *P. spinosum*, and *P. acanthophoron* were most virulent to seedlings of *Pinus patula*, in that order of importance. *E. grandis* was also highly susceptible to *P. irregulare*, *P. pyrilobum*, *P. tardicrescens*, and *Pythium* Group F. *E. grandis* does not form part of the old-lands problem, but it is an important forestry species in other parts of the country. It was included for comparative purposes and to determine whether it could be used as an alternative species on the previously cultivated lands. The very high degree of susceptibility of *E. grandis* was probably unusual and could be ascribed to the fact that young seedlings were used, which are more susceptible to *Pythium* infection (7,13).

The land preparation technique involving ridging was chosen because of the mountainous terrain. This approach to site preparation appeared to contribute to the mortality of plants during establishment. Results of this study showed that populations of *P. irregulare* were highest on the ridges where seedlings were planted. *P. irregulare* is known to be most common in the top 20 cm of soil (10,15), and this soil was used to prepare the planting ridges. The low populations of *P. irregulare* in soil adjacent to the planting ridges can thus be explained by the removal of the top soil layer to prepare the planting ridges. An alternative planting technique, chosen to reduce the inoculum levels, would probably result in a lower disease incidence (2).

Ridging was also used for water conservation in the root zones of the seedlings. However, excessive infiltration of water into the subsoil excludes any storage of water in the topsoil (27). Therefore, it was believed that the influence of *Pythium* spp. on the performance of seedlings would be of minor importance (22), since *Pythium* spp. are generally associated with waterlogged conditions (3,4,16,24). However, virulence of *P. irregulare* on peach was unaffected by waterlogged conditions, because sporangia did not produce zoospores, but formed germ tubes (4). Waterlogging seems, therefore, not to be an essential component for disease expression by *P. irregulare*.

Problems with establishing *Pinus patula* on previously cultivated lands is a serious impediment for the South African forestry industry. In this study, it was shown that *P. irregulare* is associated with these deaths. At this stage, there is no indication that edaphic factors are associated with this problem. However, it is a well-known fact that root diseases are of complex nature and often involve more than one pathogen (6–8,13, 17,18,23). Abiotic factors such as nutrient deficiencies or imbalances, waterlogging, microbial populations of the soil, and soil structure (5,7,11,19,31,38) can also be important. Factors contributing to infection of plants by *Pythium* spp. should, therefore, receive urgent attention.

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