Phytophthora clandestina, Cause of Severe Taproot Rot of Subterranean Clover in Victoria, Australia

F. C. GREENHALGH, Plant Pathologist, Plant Research Institute, Swan Street, Burnley, Australia, 3121, and P. A. TAYLOR, Plant Pathologist, Irrigation Research Institute, Tatura, Australia, 3616

ABSTRACT

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Surveys showed that the recently discovered fungus *Phytophthora clandestina* is widely distributed and consistently associated with rotted taproots of subterranean clover in dryland and irrigated pastures of Victoria. Under controlled environmental conditions, *P. clandestina* caused extensive taproot rot of subterranean clover in pasteurized and untreated sandy loam at either continually high water potentials (0 to −1/3 bar) or alternating high and low (−3 to −5 bar) potentials. The fungus also rotted taproots of plants in pasteurized clay loam and was the probable cause of the disease in untreated clay loam at high water potentials. *Fusarium avenaceum* and *Pythium irregulare* did not interact with *P. clandestina* or cause significant disease in the untreated soils. In addition, the symptoms that these fungi caused on roots in the pasteurized soils were different from those commonly observed in the field. Therefore, the role of *F. avenaceum* and *Pythium irregulare* in the etiology of root rots of subterranean clover is questioned.

Subterranean clover (*Trifolium subterraneum* L.) is the most important pasture legume in temperate regions of southern and eastern Australia (12). It is grown on more than 20×10^6 ha of mainly dryland pasture to provide protein for livestock and to improve soil fertility (4). This annual plant has been utilized successfully in Australian agriculture since the 1920s (11), but during the last 15–20 yr, productivity of the legume has declined in many areas (3,6).

Root diseases have been associated with the decline of subterranean clover in parts of New South Wales (14), Western Australia (1), and Victoria (2,8,10), and studies implicated *Fusarium avenaceum* (Corda ex. Fr.) Sacc. and *Pythium irregulare* Buisman as causal agents. These conclusions on the etiology of root rots were based mostly on results of pathogenicity tests in either sand or steam-air-treated soils and potting mixes. Investigations by Greenhalgh and Lucas (7), however, showed that severity of root rots caused by *F. avenaceum* and *Pythium irregulare* was markedly reduced in untreated soils from pastures of Victoria. This demonstrated the need for care when interpreting data from experiments with pasteurized soils and also suggested that pathogens other than *F. avenaceum* and *Pythium irregulare* may cause root rots.

Recently, a new species of *Phytophthora*, *P. clandestina* Taylor, Pascoe, & Greenhalgh (17), was isolated from rotted roots of subterranean clover from irrigated pastures of Victoria (15). Preliminary tests showed that *P. clandestina* is pathogenic to roots of subterranean clover, and therefore its role in the etiology of root rots requires clarification, especially if the fungus is widely distributed in pastures of Victoria. This study investigates the association of *P. clandestina* with rotted roots of subterranean clover in dryland and irrigated pastures of Victoria. It also compares the virulence of *F. avenaceum*, *P. clandestina*, and *Pythium irregulare*, alone and in combinations, in pasteurized and untreated soils under controlled environmental conditions.

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MATERIALS AND METHODS

Surveys. The occurrence of *P. clandestina* on roots of subterranean clover in pastures of Victoria was studied in June and July 1983. Plants with severely rotted taproots were collected from 82 dryland pastures at widespread locations in either western, northern, northeastern, or southeastern Victoria and from 23 irrigated pastures in northern Victoria. Roots of five plants from each site were washed with tap water, placed in sterile distilled water in petri dishes for 48 hr at 18 C, and examined microscopically for sporangia of *P. clandestina* (17).

In a second survey in May 1984, the association of *P. clandestina* with rotted roots was studied in more detail. Plants with taproots in early stages of necrosis were collected from an irrigated pasture at Numurkah in northern Victoria and from two dryland pastures, one at Harcourt in northern Victoria and the other at Flinders in southern Victoria. Roots of 50 plants from each site were examined for *P. clandestina* by the method described.

Tests for virulence. The virulence of *F. avenaceum*, *P. clandestina*, and *Pythium irregulare*, alone and in combinations, was tested in a clay loam (pH 6.2) from an irrigated pasture at Numurkah and in a sandy loam (pH 5.4) from a dryland pasture at Clunes in western Victoria. The soils were from sites with histories of root rots of subterranean clover. Outbreaks of the diseases occurred 2 yr before the sandy loam was collected and in each of 3 yr before the clay loam was sampled. Soil from the surface 15 cm of each pasture was sieved (4-mm mesh) and either treated with a steam-air mixture at 60 C for 60 min or left untreated.

The isolate of *P. clandestina* described by Taylor et al (17) and isolates of *Pythium irregulare* and *F. avenaceum* from roots of subterranean clover were used in the tests. The isolates of *Pythium irregulare* and *F. avenaceum* were selected because they were highly pathogenic to roots of subterranean clover in sand according to a fungal disk test (10). The fungi were grown on fine-grade vermiculite after it was moistened with lima bean broth (2 L of broth per 1 kg of vermiculite) and autoclaved at 121 C for 20 min. The broth was prepared by blending 200 g of canned lima beans (Masterfoods) in 1 L of distilled water and straining the homogenate through cheesecloth. *F. avenaceum* and *Pythium irregulare* were grown for 2 wk, and *P. clandestina*, for 4 wk on the amended vermiculite at 25 C.

The vermiculite colonized by each fungus was uniformly incorporated into the untreated and pasteurized soils 7 days after the steam-air treatment. Each fungus, either alone or in combined treatments, was introduced into the soils at a rate of 1% (w/w). Uninfested vermiculite moistened with lima bean broth was added to the soils at either 1 or 3% for control treatments.

Thirty seeds of subterranean clover cultivar Mt. Barker were planted 2 mm deep in amended soil in each pot (10 cm in diameter × 10 cm high). Previous tests established that the seed germinated at a rate of 83% and was free from contamination by *F. avenaceum*. After sowing, soil was wetted with distilled water to matric potentials between 0 and −1/3 bar, and pots were placed in a controlled-environment cabinet at 20 C

and with a 12-hr photoperiod (120 $\mu E/m^2/s$). Relative humidity was maintained at 100% for the first 5 days, then reduced to 80 ± 5%. Treatments were replicated five times in a randomized block design.

After seedling emergence, soil-water potentials were maintained at high levels (0 to −1/3 bar) in tests with the clay and sandy loams. In a subsequent test with the sandy loam, water potentials were alternated between high (0 to −1/3 bar) and low (−3 to −5 bar) levels. The drying cycle was included in this test because low water potentials have been reported to favor development of diseases caused by *Fusarium* spp. (5). The high potentials were monitored with hydraulic tensiometers (13), and the low potentials, with Wescor PT51 psychrometers and an HR-33 Dew Point Microvoltmeter (19). The psychrometer sensors and tensiometers were installed 4 cm deep in the soil.

Six weeks after sowing, the number of plants was recorded and severity of root rot on each plant was rated on a scale of 0–5 (7) (Table 1). In addition, 30 plants with severely rotted roots were selected from each treatment and roots of 10 plants were examined in sterile distilled water for sporangia of *P. clandestina* as described. The taproots of the other plants were dipped in 70% ethanol, washed in sterile water, and blotted dry. Segments (1–3 mm) of taproots of 10 plants were placed on $P_{10}VP$ medium (18), and those of the remaining 10 plants were placed on PCNB/Achromycin medium (9) and incubated at 25 C. After 5 days, *Pythium irregulare* was identified on $P_{10}VP$ medium, and fungi on PCNB/Achromycin medium were transferred onto potato-sucrose agar to identify *F. avenaceum*.

RESULTS

Surveys. In the extensive survey in 1983, *P. clandestina* was detected in roots of subterranean clover from 29 of 35 pastures sampled in the western region, from 9 to 15 sites in the northeastern district, and from 13 of 21 sites in southeastern Victoria. It was also detected in roots from 21 of the 23 irrigated pastures and from all 11 dryland pastures sampled in northern Victoria.

In the intensive survey in 1984, *P. clandestina* was detected in 70, 76, and 86% of rotted taproots from Harcourt, Flinders, and Numurkah, respectively.

Tests for virulence. *F. avenaceum* and *Pythium irregulare*, either individually or in combined treatments, reduced ($P = 0.01$) plant numbers in pasteurized but not in untreated clay loam (Table 1). *P. clandestina* did not affect plant numbers in the pasteurized or untreated soil. In the pasteurized soil, root rot severity was greatest when *P. clandestina* was present alone or in combination with *F. avenaceum* or *Pythium irregulare*. In untreated soil, however, none of the treatments affected root rot severity, which was comparable to that obtained with *P. clandestina* alone in the pasteurized soil.

Each fungal treatment reduced ($P = 0.05$) plant numbers in pasteurized but not in untreated sandy loam at continually high water potentials (Table 2). *P. clandestina* alone or in combinations with *F. avenaceum* and *Pythium irregulare* caused the most severe root rot in the pasteurized and untreated soil. Disease was more severe in the pasteurized than in the untreated soil.

F. avenaceum and *Pythium irregulare* alone and in combinations decreased plant numbers in pasteurized but not in untreated sandy loam under alternating moist and dry conditions (Table 3). *P. clandestina* did not affect plant numbers in the pasteurized or untreated soil. In the pasteurized soil, root rot severity was high with *P. clandestina* and greatest for the *P. clandestina* × *F. avenaceum* treatment. However, analysis of the data showed that there was no significant ($P = 0.05$) interaction between *P. clandestina* and *F. avenaceum*. *P. clandestina* alone or in combined treatments caused the most severe root rot in the untreated soil, but disease

Table 1. Individual and combined effects of *Fusarium avenaceum, Phytophthora clandestina,* and *Pythium irregulare* on severity of root rot and number of subterranean clover plants in pasteurized and untreated clay loam at continually high water potentials[a]

Fungi introduced into soil	Number of plants[b]		Root rot severity[c]	
	Pasteurized	Untreated	Pasteurized	Untreated
F. avenaceum	17.0	19.6	0.9	2.2
Pythium irregulare	11.8	18.2	1.8	2.1
P. clandestina	22.8	21.8	2.7	2.4
F. avenaceum + *Pythium irregulare*	1.2	20.6	ND[d]	2.3
P. clandestina + *Pythium irregulare*	14.8	21.2	2.2	2.3
P. clandestina + *F. avenaceum*	15.6	21.8	2.9	2.3
P. clandestina + *F. avenaceum* + *Pythium irregulare*	3.4	19.6	ND	2.4
Uninoculated controls				
1% Level of vermiculite	23.2	20.6	0.1	2.0
3% Level of vermiculite	23.0	22.2	0.1	2.2
LSD between any two values				
$P = 0.05$	2.8		0.6	
$P = 0.01$	3.7		0.8	

[a] Fungi on vermiculite moistened with lima bean broth were introduced into either pasteurized (60 C for 60 min) or untreated soil, which was then maintained at water potentials between 0 and −1/3 bar. Values represent means of five replicates and were obtained 6 wk after seeds were sown in pots in a growth cabinet at 20 C.
[b] An average of 25 germinable seeds were sown in each pot.
[c] Root rot severity was rated on a scale of 0–5, where 0 = roots healthy, 1 = slightly lateral root rot, 2 = moderately severe lateral root rot or slight taproot rot or both, 3 = severe lateral root rot or moderately severe taproot rot or both, 4 = severe taproot rot, and 5 = plant dead.
[d] Root rot severity was not determined because of low number of plants.

Table 2. Individual and combined effects of *Fusarium avenaceum, Phytophthora clandestina,* and *Pythium irregulare* on severity of root rot and number of subterranean clover plants in pasteurized and untreated sandy loam at continually high water potentials[a]

Fungi introduced into soil	Number of plants[b]		Root rot severity[c]	
	Pasteurized	Untreated	Pasteurized	Untreated
F. avenaceum	13.2	21.6	1.3	0.7
Pythium irregulare	9.8	18.0	2.8	1.7
P. clandestina	13.4	17.0	4.6	2.8
F. avenaceum + *Pythium irregulare*	10.0	20.0	2.6	0.7
P. clandestina + *Pythium irregulare*	3.2	16.0	4.9	2.8
P. clandestina + *F. avenaceum*	11.6	16.8	4.5	2.9
P. clandestina + *F. avenaceum* + *Pythium irregulare*	5.8	16.2	4.7	3.1
Uninoculated controls				
1% Level of vermiculite	18.4	20.0	0.1	0.5
3% Level of vermiculite	19.8	21.6	0.5	1.3
LSD between any two values				
$P = 0.05$	4.0		0.5	
$P = 0.01$	5.3		0.7	

[a] Fungi on vermiculite moistened with lima bean broth were introduced into either pasteurized (60 C for 60 min) or untreated soil, which was then maintained at water potentials between 0 and −1/3 bar. Values represent means of five replicates and were obtained 6 wk after seeds were sown in pots in a growth cabinet at 20 C.
[b] An average of 25 germinable seeds were sown in each pot.
[c] Root rot severity was rated on a scale of 0–5, where 0 = roots healthy, 1 = slightly lateral root rot, 2 = moderately severe lateral root rot or slight taproot rot or both, 3 = severe lateral root rot or moderately severe taproot rot or both, 4 = severe taproot rot, and 5 = plant dead.

Table 3. Individual and combined effects of *Fusarium avenaceum, Phytophthora clandestina*, and *Pythium irregulare* on severity of root rot and number of subterranean clover plants in pasteurized and untreated sandy loam at alternating high and low water potentials[a]

Fungi introduced into soil	Number of plants[b]		Root rot severity[c]	
	Pasteurized	Untreated	Pasteurized	Untreated
F. avenaceum	16.6	19.2	0.3	0.7
Pythium irregulare	14.8	19.6	1.6	0.7
P. clandestina	21.4	20.6	4.2	2.8
F. avenaceum + *Pythium irregulare*	10.8	20.4	1.4	0.7
P. clandestina + *Pythium irregulare*	17.6	17.6	4.2	2.7
P. clandestina + *F. avenaceum*	17.2	20.6	4.9	2.7
P. clandestina + *F. avenaceum* + *Pythium irregulare*	12.2	18.4	4.4	2.6
Uninoculated controls				
1% Level of vermiculite	21.4	19.8	0.2	0.5
3% Level of vermiculite	22.2	19.6	0.2	0.5
LSD between any two values				
$P = 0.05$	3.3		0.3	
$P = 0.01$	4.3		0.5	

[a] Fungi on vermiculite moistened with lima bean broth were introduced into either pasteurized (60 C for 60 min) or untreated soil, which was then subjected to a cycle of wetting (0 to −1/3 bar) and drying (−3 to −5 bar). Values represent means of five replicates and were obtained 6 wk after seeds were sown in pots in a growth cabinet at 20 C.

[b] An average of 25 germinable seeds were sown in each pot.

[c] Root rot severity was rated on a scale of 0–5, where 0 = roots healthy, 1 = slightly lateral root rot, 2 = moderately severe lateral root rot or slight taproot rot or both, 3 = severe lateral root rot or moderately severe taproot rot or both, 4 = severe taproot rot, and 5 = plant dead.

severity was less than in the pasteurized soil.

In each test, fungi introduced either alone or in combination into pasteurized and untreated soil were recovered consistently on rotted roots. None of the fungi was detected in roots in control treatments for the pasteurized soils, and *F. avenaceum* and *Pythium irregulare* were isolated infrequently from roots in the controls for the untreated soils. The frequency of detection of *P. clandestina* on roots in controls for untreated soil was low (10–30%) in the two tests with sandy loam and high (100%) in the test with clay loam.

Examination of roots in the pasteurized soils showed that each fungus caused a different type of rot. *F. avenaceum* caused a blackening of cortical tissue of the taproot immediately below the hypocotyl. *Pythium irregulare* caused a black discoloration of cortical and vascular tissues on the lower part of the taproot and brown to black lesions at the tips of lateral roots. In contrast, *P. clandestina* caused an orange-brown to dark brown discoloration of cortical tissue and the stele on the upper part of the taproot. The lesion severed the taproot and occasionally extended into the hypocotyl.

DISCUSSION

The study indicates that the etiology of root rots of subterranean clover in Australia needs to be reappraised. The widespread distribution of *P. clandestina* and its virulence on taproots in untreated soil suggest that this newly discovered fungus is the cause of severe taproot rot in Victoria. Results of the tests for virulence also indicate that in comparison with *P. clandestina*, *F. avenaceum* and *Pythium irregulare* are only minor pathogens of subterranean clover.

The dramatic reduction in severity of diseases caused by *F. avenaceum* and *Pythium irregulare* in untreated soils is apparently due to the biological buffering capacity of soils, which is eliminated by the pasteurization treatment. Therefore, previous results obtained with *F. avenaceum* and *Pythium irregulare* in pasteurized soils or artificial media (1,2,8,10,14) cannot be regarded as conclusive evidence that these fungi are important root pathogens of subterranean clover. The role of *F. avenaceum* and *Pythium irregulare* is also questioned because they did not interact with *P. clandestina* or cause symptoms on roots similar to those commonly observed in the field (16; F. C. Greenhalgh, *unpublished*).

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