Pythium spp. Associated with Rooibos Seedlings, and Their Pathogenicity Toward Rooibos, Lupin, and Oat

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Abstract

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Rooibos (*Aspalathus linearis*) is an important indigenous crop in South Africa. Oomycetes are a common problem in rooibos nurseries, causing serious losses, but limited information is available on the species involved. Molecular and morphological analyses of 117 oomycete isolates from 19 rooibos nurseries and 33 isolates from 11 native rooibos sites revealed the presence of several *Pythium* spp., including *Pythium acanthicum*, *P. irregulare*, *P. mamillatum*, *P. myriotylum*, *P. pyrilobum*, *P. cederbergense*, and *Pythium* RB II, and *Phytophthora cinnamomi* (native site). Most of the species were identified in nurseries and native rooibos, with *Pythium irregulare* being the most common species occurring in all nurseries and 46% of the native sites. Phylogenetic analyses of the internal transcribed spacer region of the *P. irregulare* isolates showed that isolates within this species complex fit

into three subclades, of which only two have previously been reported. On rooibos, all species except *P. acanthicum* and the previously characterized *P. cederbergense* and *Pythium* RB II were pathogenic and highly virulent. On lupin and oat, rotation crops in nurseries, the three aforementioned species were also nonpathogenic. All the other oomycete species were pathogenic on lupin but less so than on rooibos. On oat, only *P. irregulare*, *P. myriotylum*, and *P. pyrilobum* were pathogenic. This is the first report of *P. mamillatum*, *P. pyrilobum*, and *P. myriotylum* as pathogens of lupin, and *P. irregulare* and *P. pyrilobum* as pathogens of oat. The three nonpathogenic *Pythium* spp. were able to significantly reduce disease caused by pathogenic species in the less susceptible lupin and oat but not on rooibos. On lupin, the nonpathogenic species enhanced the virulence of *Phytophthora cinnamomi*.

The rooibos (*Aspalathus linearis* (N.L. Burm.) R. Dahlgr.) plant, which is well known for being used to produce herbal tea and a few other products, is only grown in the Cederberg Mountains in South Africa (27). The first step in rooibos production consists of seed being sown in open-bed nurseries in sandy soils, which are kept at field capacity. Approximately 4 months after sowing, the emerged seedlings are transplanted into commercial open-field production systems. In nurseries, special production practices are required for cultivating rooibos, because it is difficult to establish and grow seedlings. One of the main factors that affects establishment in nurseries is damping-off, which occurs within the first 6 weeks of rooibos seedling growth. Damping-off can cause serious losses in nurseries, generally in the order of 50% or more if no control measures are applied. Several plant pathogens, including *Pythium*, *Fusarium*, and *Rhizoctonia* spp., cause damping-off, with species within the genus *Pythium* being among the most prevalent and important pathogens (*unpublished data*).

The genus *Pythium* belongs to the phylum Oomycota, commonly known as oomycetes. Species within this genus are common in soils and have been reported from all over the world (15,68). Many species are important soilborne plant pathogens that cause serious yield losses on a wide range of agricultural crops, including cereals, vegetables, shrubs, fruit trees, and ornamentals (6,23, 38,73). However, several species are saprophytic, with some

species even enhancing plant growth and showing potential as biocontrol agents (37,68). For example, *Pythium acanthicum* Drechsler, *P. oligandrum* Drechsler, and *P. periplocum* Drechsler are effective in suppressing damping-off caused by *P. ultimum* Trow, *P. irregulare* Buiman, *P. mamillatum* Meurs, *P. myriotylum* Drechsler, and *P. vexans* de Bary (1,36,55,68). *P. oligandrum* is also antagonistic toward *Phytophthora parasitica* Dastur (51), *Rhizoctonia solani* Kühn and *Fusarium culmorum* (W.G. Sm.) Sacc. (11). On apple (*Malus domestica* Borkh.), several undescribed *Pythium* spp. (*Pythium* MM1, *Pythium* MM3 [aff. *oedocheilum*], and *Pythium* MM5 [aff. *vexans*]) have shown biocontrol potential, because they were able to suppress root rot on apple seedlings caused by *Pythium sylvaticum* Campb. & F.F. Hendrix and *P. ultimum* (40).

In South Africa, rooibos growers must use an integrated management approach for managing *Pythium* damping-off, where several cultural practices, biological control, and fungicides could potentially be combined. Several fungicides (metalaxyl, metalaxyl-M, mancozeb, thiram, captab, dazomet, and dichlorophen) are registered in South Africa for the management of *Pythium* diseases (46), of which metalaxyl-M is known to be very effective (59). However, fungicides may not be applied in organic rooibos production systems and, therefore, only biological control and cultural practices (water management, fertilizer applications, organic amendments, and crop rotation) may be used (16,25,28,42,45). Of the cultural practices, water management is very important because excess water and soil moisture favors *Pythium* spp. reproduction and infection (20). Rotation crops and organic amendments can sometimes be effective at suppressing soilborne diseases, including those caused by *Pythium* spp. (10,17,24), but little is known about their effect in rooibos production systems and whether rotation crops such as lupin and oat are hosting rooibos soilborne pathogens. Biological control also has potential for the management of *Pythium* damping-off in some crop systems (21,35,45,47,54,71) but its potential has not yet been investigated in rooibos. The only

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*The e-Xtra logo stands for “electronic extra” and indicates that two additional tables are available in the online edition.

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biocontrol agent that is registered for *Pythium* root rots in South Africa is *Trichoderma harzianum* Rifai (N. Mkize, Department of Agriculture, Forestry and Fisheries, directorate Food Safety and Quality Assurance, South Africa, *personal communication*).

The genus *Pythium* consists of more than 106 species that can be identified using morphological and DNA data (60). At the DNA level, *Pythium* spp. can be identified through polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) regions, followed by PCR restriction fragment length polymorphisms (RFLPs) or sequencing (34,41). Phylogenetic analysis of ITS sequence data has revealed the presence of 11 phylogenetic clades (A to K) within the genus (34). The validity of several species within some of these clades has been questioned. For example, in clade F, Garzón et al. (19) identified two distinct species, *P. cryptoirregulare* and *P. irregulare* s.s., within the *P. irregulare* species complex. However, Spies et al. (63) provided evidence suggesting that all currently described species within the *P. irregulare* complex (*P. cryptoirregulare*, *P. cylindrosporum*, *P. irregulare*, and *P. regulare*) form a single phylogenetic species that is genetically diverse.

Very little is known about the specific *Pythium* spp. and other oomycetes such as *Phytophthora* that are associated with rooibos and their pathogenicity toward rooibos and two nursery rotation crops (lupin [*Lupinus angustifolius* L.] and oat [*Avena sativa* L.]). Previously, only two *Pythium* clade G taxa (*P. cederbergense* and *Pythium* RB II) were identified from rooibos, which were shown to

be nonpathogenic toward rooibos, lupin, and oat (2). Knowledge of the specific oomycete species involved and their pathogenicity is important, because this will allow for the future development of molecular detection and quantification methods, which can be used to monitor the effectiveness of different management strategies. The aims of our study were to determine (i) the oomycete species associated with rooibos seedlings in 19 nurseries, (ii) whether the species in native rooibos ecosystems (mature plants in native ecosystem) are similar to those in nurseries, (iii) the pathogenicity of oomycete species toward rooibos and two rotation crops (lupin and oat), and (iv) whether nonpathogenic oomycete species could suppress disease caused by pathogenic species on rooibos, lupin, and oat (i.e., if a biological control effect was evident).

Materials and Methods

Survey and isolations from rooibos nurseries and native ecosystems. Rhizosphere soils from 19 rooibos nurseries were sampled from a depth of 20 cm in March and April and in June and July 2007 to 2009. Rhizosphere soils were also collected from rooibos plants in 11 native ecosystems (Table 1). The rhizosphere soils were collected by uprooting the plants and vigorously shaking the roots, which allowed collection of the soil adhering to the roots. The soil was used in a glasshouse trial, where rooibos seed were sown in the different soils and kept at temperatures of approximately 18°C (night) and 27°C (day). After 4 weeks, the roots of damped-off seedlings were plated onto *Pythium* and *Phytophthora*

Table 1. Occurrence of oomycete species (*Pythium* and *Phytophthora*) in rooibos nurseries and native rooibos

Location ^y	Percent isolates (number of isolates) ^x							
	<i>P. cinn</i>	<i>P. aca</i>	<i>P. irregulare</i> ^z	<i>P. mam</i>	<i>P. myr</i>	<i>P. pyr</i>	<i>P. ced</i>	RB II
Nursery								
A	0	8 (1)	8 (1) PY1, 46 (6) PY2, 8 (1) PY4, 22 (3) PY8	0	8 (1)	0	0	0
B	0	0	11 (1) PY2, 45 (4) PY4, 33 (3) PY5	0	0	0	0	11 (1)
C	0	0	5 (1) PY1, 29 (5) PY2, 56 (10) PY4, 5 (1) PY5	0	0	0	0	5 (1)
D	0	0	8 (1) PY1, 17 (2) PY2, 25 (3) PY4, 50 (6) PY8	0	0	0	0	0
E	0	0	44 (3) PY1, 14 (1) PY2, 14 (1) PY4, 14 (1) PY8, 14 (1) PY11	0	0	0	0	0
F	0	0	25 (1) PY4, 25 (1) PY8, 50 (2) PY11	0	0	0	0	0
G	0	0	20 (1) PY1, 80 (4) PY2	0	0	0	0	0
H	0	0	100 (5) PY4	0	0	0	0	0
I	0	17 (1)	17 (1) PY11	17 (1)	49 (3)	0	0	0
J	0	0	33 (1) PY4	67 (2)	0	0	0	0
K	0	0	33 (2) PY4	0	67 (4)	0	0	0
L	0	0	33 (1) PY2, 33 (1) PY4	0	33 (1)	0	0	0
M	0	0	100 (1) PY2	0	0	0	0	0
N	0	0	7 (1) PY2, 7 (1) PY5, 7 (1) PY11	0	50 (7)	0	22 (3)	7 (1)
O	0	0	40 (2) PY1, 40 (2) PY2, 20 (1) PY4	0	0	0	0	0
P	0	0	100 (1) PY4	0	0	0	0	0
Q	0	0	100 (1) PY4	0	0	0	0	0
R	0	0	33 (1) PY1, 67 (2) PY2	0	0	0	0	0
S	0	0	100 (1) PY2	0	0	0	0	0
Total	0	2 (2)	9 (10) PY1, 23 (27) PY2, 27 (32) PY4, 4 (5) PY5, 9 (11) PY8, 4 (5) PY11	3 (3)	13 (16)	0	3 (3)	3 (3)
Native								
A			33 (1) PY4				67 (2)	
B			100 (9) PY4					
C				50 (2)		50 (2)		
D	20 (1)					40 (2)	40 (2)	
E			100 (3) PY2					
F				100 (1)				
G				100 (1)				
H			100 (1) PY4					
I							100 (1)	
J			100 (1) PY2					
K						75 (3)	25 (1)	
Total	3 (1)	0	12 (4) PY2, 34 (11) PY4	12 (4)	0	21 (7)	18 (6)	0
N + N ^z	1 (1)	1 (2)	7 (10) PY1, 21 (31) PY2, 29 (43) PY4, 3 (5) PY5, 8 (11) PY8, 3 (5) PY11	4 (7)	11 (16)	4 (7)	6 (9)	2 (3)

^x *P. cinn* = *Phytophthora cinnamomi*, *P. aca* = *Pythium acanthicum*, *P. mam* = *P. mamillatum*, *P. myr* = *P. myriotylum*, *P. pyr* = *P. pyriforme*, *P. ced* = *P. cederbergense*, and RBII = *Pythium* RB II.

^y Native = Native population and N + N = total nursery plus native.

^z Polymerase chain reaction restriction fragment length polymorphism group (PY 1, 2, 4, 5, 8, and 11) to which *P. irregulare* isolates belonged to are indicated.

selective media (PARP and PARPH, respectively; 6,26). Isolations from glasshouse-grown roots were more effective for isolating pathogens compared with isolations that were made from field-grown plant roots, where many saprophytic and secondary microbes from old roots interfered with pathogen isolation (*unpublished data*). Hyphae emerging from the plated roots were hyphal tipped to cornmeal agar (CMA; Sigma Aldrich), followed by a second hyphal tipping. All isolates were stored as CMA culture plugs in sterile deionized water containing grass blades, as V8-agar (18) plugs in sterile deionized water, and on potato-carrot agar (14) slant cultures at 15°C.

Molecular species identification. Oomycete isolates were grown on V8 agar for 5 to 7 days. Aerial mycelium was harvested and genomic DNA was extracted from a total of 150 isolates using a slight modification of the cetyltrimethylammonium bromide method of Lee and Taylor (32), as previously described (67).

PCR-RFLP analyses. The ITS region of the isolates was amplified from DNA using primers ITS6 (7) and ITS4 (72). The PCR reaction consisted of 1× PCR buffer (Bioline), 2.5 mM MgCl₂, 0.2 mM dNTPs, 0.65 U of BIOTAQ (Bioline), bovine serum albumin Fraction V (Roche Diagnostics South Africa) at 0.05 mg/ml, and 0.2 mM each primer in a total reaction volume of 40 µl. Amplifications were performed in a 2720 Applied Biosystems thermal cycler with an initial denaturation at 94°C for 5 min; followed by 32 cycles of 94°C for 30 s, annealing for 30 s at 55°C, and extension at 72°C for 90 s; and 1 cycle of extension at 72°C for 7 min. PCR products were resolved in 1% agarose gels and DNA fragments were visualized by staining in a solution of ethidium bromide. Successfully amplified PCR products were restriction digested with enzymes *Hinf*I and *Hha*I in a single reaction (41), according to the manufacturer's instructions (Fermentas Inc.). Because some of the PCR-RFLP patterns, such as Py1 versus Py4 and Py8 versus Py11, were not easily differentiated from each other based on the *Hinf*I and *Hha*I digest (see Results section) due to light bands not always being reproducible, the PCR products of isolates belonging to these PCR-RFLP groups and all groups identified as *P. irregulare* were also digested using only enzyme *Hha*I. This allowed for a more accurate identification of the *P. irregulare* PCR-RFLP groups when used in conjunction with the *Hinf*I and *Hha*I data. The PCR-RFLP products were run on a 3% agarose gel, and isolates that had the same RFLP pattern were assigned to the same RFLP group.

ITS sequencing. The ITS regions of at least two isolates of each PCR-RFLP group were sequenced and double-strand consensus sequences were obtained, as previously described (41). The sequences were submitted to BLAST analyses in GenBank, and identified to species level using sequences submitted by Lévesque and De Cock (34).

Phylogenetic analyses of *P. irregulare*. The genetic diversity among the *P. irregulare* isolates was analyzed phylogenetically. Two to three isolates representative of each of the six *P. irregulare* PCR-RFLP patterns (see Results) were selected for sequencing. The PCR product of one of the isolates (R8282W1) was cloned and sequenced, as previously described (63), because direct sequencing yielded poor quality sequence data. The ITS consensus sequences were submitted to GenBank (KC855063, KC855064, KC855065, KC855066, KC855067, KC855068, KC855069, KC855070, KC855071, KC855072, KC855073, KC855074, KC855075, and KC855076). The reference sequences used in the analyses included species that fit into *Pythium* clade F and sequences representative of all the *P. irregulare* subclades identified by Spies et al. (63).

Maximum parsimony analyses of *Pythium* clade F was conducted as previously described (63). Bayesian analyses were conducted using MrBayes v. 3.0b4 (58) and the program MrModeltest (53) was used to select the optimal model of sequence evolution, as previously described (44). The selected model for the ITS region was HKY, with an estimated proportion of invariable sites (+I) and an estimated γ parameter (+G). The first 433,100 generations (burn-in) were discarded for the ITS analysis.

Morphological identification of oomycete species. Morphological identification of all *Pythium* isolates were conducted as previously described (44). *Phytophthora* spp. were identified using Erwin and Ribeiro (16).

Pathogenicity assays. A subset of 32 isolates was selected for inclusion in pathogenicity assays on lupin, oat, and rooibos. The isolates represented all the different species that were identified from nurseries and the native ecosystems (Table 1), except for *P. cederbergense* and *Pythium* RB II that were not included in the pathogenicity studies because the pathogenicity of these groups has previously been established (2). The selected isolates included one *Phytophthora cinnamomi* isolate, two isolates of *Pythium acanthicum*, 16 isolates of *P. irregulare* that represented all six PCR-RFLP groups identified for this species (see Results), three *P. mamillatum* isolates, six isolates of *P. myriotylum*, and four isolates of *P. pyrilobum*.

Pathogenicity assays were conducted in a glasshouse pot trial. A pasteurized planting medium (equal parts of soil, perlite, and sand) was inoculated with sand and wheat bran inoculum of each of the oomycete isolates at a concentration of 0.05% inoculum/planting medium (wt/wt). The sand-wheat bran inoculum was prepared as previously described (2,30). The control consisted of planting medium inoculated with sand-wheat bran that was inoculated with uncolonized agar plugs. Plastic pots (13 cm in diameter) were filled with 800 g of the inoculated media. Ten planting holes were made for seed in each pot to a depth of 1.5 cm (rooibos) to 2 cm (lupin and oat). The number of seed planted per pot was 50 for rooibos and oat and 20 for lupin. Three replications were included for each treatment and the trial was conducted twice. The pots were watered almost on a daily basis in order to keep the soil at field capacity. The experiments were carried out in the glasshouse at temperatures of approximately 18°C (night) and 27°C (day).

Pathogenicity of the isolates was determined by evaluating seedling length and percent survival. The survival of seedlings in all three crops was evaluated 2 weeks after planting. The seedling length of the surviving seedlings was measured for lupin and oat 2 weeks after planting but, for rooibos, it was only evaluated after 1 month, because rooibos is a very slow grower and only cotyledons are present 2 weeks after planting. Roots were not evaluated for rot, because previous studies showed that the analysis was too subjective, and that seedling length and survival provided more accurate data (*unpublished data*). Reisolations were made from the roots, if available, or from damped-off seed to fulfill Koch's postulates (67).

Evaluating the potential of nonpathogenic *Pythium* spp. to suppress damping-off caused by pathogenic oomycete species.

The ability of nonpathogenic *Pythium* spp. to suppress damping-off caused by pathogenic oomycete species was assessed on rooibos, lupin, and oat using a sand-wheat bran inoculation method, as described above. The oomycete species that were used included all the species that were identified from rooibos in the current study (Table 1). Each species was represented by only one isolate and included the nonpathogenic species *P. acanthicum* (R8206Q), *Pythium* RB II (STE-U 7548), and *P. cederbergense* (STE-U 7555) and the pathogenic species *Phytophthora cinnamomi* (R8303A), *Pythium irregulare* (R8308F), *P. mamillatum* (R8201C), *P. myriotylum* (R8197F), and *P. pyrilobum* (R8198A) (see Results section). The isolates that were used to represent pathogenic species were all pathogenic toward lupin and rooibos, whereas only *P. irregulare* (R8308F), *P. myriotylum* (R8197F), and *P. pyrilobum* (R8298A) were pathogenic toward oat (see Results section). In each mixed-species inoculation treatment (M1 to M7), each isolate in the mixture was first inoculated separately into sand-wheat bran medium and, after 10 days of growth, the colonized sand-wheat bran of different species was co-inoculated into the planting medium. Each isolate in the mixture was inoculated at a concentration of 0.05% (wt/wt). The trial included seven treatments and three replicates per treatment and was conducted twice. The seven treatments and the specific species used in the co-inoculations consisted of M1, a mixture of pathogenic oomycetes spp. (*Phy-*

Phytophthora cinnamomi, *Pythium irregulare*, *P. mamillatum*, *P. myriotylum*, and *P. pyriformis*) and nonpathogenic *Pythium* spp. (*P. acanthicum*, *P. cederbergense*, and *Pythium* RB II); M2, a mixture of only pathogenic oomycete species (*Phytophthora cinnamomi*, *Pythium irregulare*, *P. mamillatum*, *P. myriotylum*, and *P. pyriformis*); M3, a mixture of only nonpathogenic *Pythium* spp. (*P. acanthicum*, *P. cederbergense*, and *Pythium* RB II); M4, a mixture of *P. irregulare* and nonpathogenic *Pythium* spp. (*P. acanthicum*, *P. cederbergense*, and *Pythium* RB II); M5, a mixture of *Phytophthora cinnamomi* and nonpathogenic *Pythium* spp. (*Pythium acanthicum*, *P. cederbergense*, and *Pythium* RB II); M6, only *P. irregulare*; and M7, only *Phytophthora cinnamomi*. The control consisted of uncolonized sand-bran medium inoculated at 0.05% (wt/wt) into the planting medium.

Statistical analyses. The plant length and percent survival data were subjected to an appropriate analysis of variance for both trials. Levene's test was conducted for homogeneity of trials (33) and, finally, the data of the two trials were combined and subjected to analysis of variance. The means of significant source effects were compared using Student's *t*-LSD (*t* least significant difference) at a 5% significance level. The Shapiro-Wilk test was performed on standardized residuals to test for deviations from normality (62). All of the above procedures were performed by SAS statistical software (version 9.2; SAS Institute Inc.).

Results

Survey and isolations from rooibos nurseries and native ecosystems. Oomycete isolates, mainly *Pythium* spp., were obtained from all the surveyed nursery and native rooibos soils. The only other oomycete genus that was identified was *Phytophthora*, which was represented by one isolate from native rooibos. In total, 150 oomycete isolates, 117 from the 19 nurseries and 33 from the 11 native ecosystems, were obtained and characterized further.

Molecular and morphological identification of oomycete species. ITS-PCR-RFLP analyses using *HinfI* and *HhaI* revealed the presence of 14 different groups (PY1 to PY14; Table 1; Fig. 1). Some of the light bands characteristic for some of the Py groups, such as PY1 versus PY4 and PY 5 versus PY 8 (Fig. 1), were not always readily differentiated and visible on gels but enzyme digestion using only *HhaI* was helpful in differentiating these groups from each other (Fig. 2). GenBank BLAST analyses of the ITS sequences of representative isolates of each of the PCR-RFLP groups showed that *Pythium irregulare* was represented by six PCR-RFLP groups (PY1, 2, 4, 5, 8, and 11), PY3 was *P. myriotylum*, PY6 was *P. mamillatum*, PY7 was *P. pyriformis*, PY9 was *P. cinnamomi*, PY10 was *P. acanthicum*, *P. cederbergense* was PY 12, and *Pythium* RB II was represented by two groups (PY 13 and 14). For the PCR-RFLP groups of *P. irregulare*, PY4 (29%) and PY2 (21%) were most abundant, followed by PY8 (8%), PY1 (7%),

PY5 (3%), and PY11 (3%) (Table 1). Morphological analyses of all the oomycete isolates corresponded to the molecular identifications.

In nurseries, six *Pythium* taxa were identified, with *P. irregulare* being isolated from all the nurseries and dominating populations, comprising 76% (90/117) of all nursery isolates (Table 1). The next most abundant species in nurseries were *P. myriotylum* (13%), followed by *P. mamillatum* (3%), *P. cederbergense* (3%), *Pythium* RB II (3%), and *P. acanthicum* (2%). In contrast to native rooibos where *P. pyriformis* was one of the prevalent species, this species and *Phytophthora cinnamomi* were not detected in nurseries (Table 1).

The species composition of *Pythium* populations differed among the 19 nurseries (Table 1). The highest species diversity was found in nurseries I and N (four species each). In nursery N, *Pythium irregulare*, *P. myriotylum*, *P. cederbergense*, and *Pythium* RB II were identified, whereas nursery I contained *P. acanthicum*, *P. irregulare*, *P. mamillatum*, and *P. myriotylum*. In both nurseries, *P. myriotylum* was the dominating (approximately 50%) species. *Pythium* populations in nursery K consisted of *P. irregulare* and *P. myriotylum* and were also dominated by *P. myriotylum*. *P. irregulare* was detected in all the nurseries, whereas *P. myriotylum* was only identified in five nurseries. The lowest number of *Pythium* isolates was detected in nurseries M, P, Q, and S, where only one isolate of *P. irregulare* was recovered in each nursery. In most (58%) of the nurseries, *P. irregulare* was also the only *Pythium* sp. detected. Furthermore, in all nurseries where more than two *P.*

Fig. 2. Six polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) groups (PY1, PY2, PY4, PY5, PY8, and PY11) that were identified among *Pythium irregulare* isolates from rooibos. The PCR-RFLP groups were identified by conducting a single restriction digestion with *HhaI* on PCR-amplified products of the internal transcribed spacer regions. A 100-bp DNA ladder (L) was used to estimate the size of digested products.

Fig. 1. Fourteen polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) groups (PY1 to PY14) that were identified among oomycete (*Pythium* and *Phytophthora*) isolates obtained from rooibos. The PCR-RFLP groups were identified by conducting a double-restriction digestion with *HinfI* and *HhaI* on PCR-amplified products of the internal transcribed spacer regions. A 100 bp DNA ladder (L) was used to estimate the size of digested products.

irregulare isolates were obtained, at least two but up to five (nursery E) different *P. irregulare* PCR-RFLP groups were identified, except for nursery H, where all five isolates belonged to the same PCR-RFLP group (Table 1).

Among the 33 oomycete isolates obtained from native rooibos soils, *P. irregulare* was also the dominant (46%) species, followed by *P. pyrilobum* (21%), *P. cederbergense* (18%), and *P. mamillatum* (12%). *Phytophthora cinnamomi* (3%) was represented by only one isolate. *Pythium irregulare* was also the most common species among the different sites, occurring in 5 of the 11 sites, followed by *P. cederbergense* that was identified in 4 of the native sites. The *P. irregulare* PCR-RFLP groups identified in the native rooibos did not differ from those in the cultivated nurseries and included all five groups. In contrast to the nurseries where *P. myriotylum* was the second most abundant species, this species, along with *P. acanthicum* and *Pythium* RB II, was not identified from native rooibos (Table 1).

Phylogenetic analyses of *P. irregulare*. Maximum parsimony and Bayesian analyses of the ITS region clustered the rooibos *P. irregulare* sequences into a single well-supported clade (97% bootstrap support, 1.00 probability) containing the entire *P. irregulare* species complex (Fig. 3). This clade was further subdivided into three subclades with good bootstrap and probability support. Subclade A (87% bootstrap, 1.00 probability) corresponded to subclades 1 and 2 of Spies et al. (63) and includes GenBank sequences of *P. irregulare* CBS 250.28 (ex-neotype strain), *P. cryptoirregulare* CBS 118731 (ex-holotype strain), and 11 rooibos isolates that represented four of the rooibos PCR-RFLP groups (PY1, PY4, PY8, and PY11). The second subclade (subclade B, 100% bootstrap support, 1.00 probability) corresponded to subclade 3 of Spies et al. (63) and includes GenBank sequences of *P. cryptoirregulare* CBS 118731 (ex-holotype strain), *P. cylindrosporum* CBS 218.94 (ex-type strain), and *P. regulare* CI-34 (holotype strain) but no rooibos isolate sequences. The third subclade (subclade C, 99% bootstrap support, 1.00 probability) represents a novel *P. irregulare* subclade that was not identified by Spies et al. (63). This subclade only contained sequences of three rooibos isolates (R8177A, R8173N, and R8292AE) that all belonged to PCR-RFLP group Py5, and a GenBank *P. irregulare* sequence (HQ643604) from the study of Robideau et al. (57).

Pathogenicity assays. There was not enough evidence ($P > 0.05$) against homogeneity of variances of the two trials for seedling survival and length based on Levene's variance ratio test and, therefore, the data of the two trials could be combined. From the analysis of variance Table (Supplementary Table S1), there was strong evidence for significant isolate-crop, species-crop, and PCR-RFLP group-crop interactions for percent survival ($P < 0.0001$) and seedling length ($P < 0.0001$). Therefore, it is appropriate to calculate the *t*-LSD in order to compare the interaction means within rows and within columns.

Most oomycete isolates and species were pathogenic toward rooibos. All the isolates of *Phytophthora cinnamomi*, *Pythium irregulare*, *P. mamillatum*, *P. myriotylum*, and *P. pyrilobum* were highly virulent on rooibos, causing 100% seedling damping-off (Table 2). *P. acanthicum* was the only nonpathogenic species, because it did not cause a significant decrease in rooibos seedling survival or seedling length compared with the uninoculated control (Table 2).

Several of the *Pythium* spp. and *Phytophthora cinnamomi* were pathogenic toward lupin. *P. cinnamomi*, *Pythium irregulare*, *P. mamillatum*, *P. myriotylum*, and *P. pyrilobum* were all considered pathogenic toward lupin because some isolates within each species significantly reduced the survival of lupin seedlings (Table 2) and caused a significant reduction in seedling length compared with the uninoculated control (Table 2). However, the isolates were less virulent than on rooibos, causing on average 40 to 60% damping-off. Similar to rooibos, *P. acanthicum* was also nonpathogenic toward lupin and did not cause a significant effect on the survival or length of lupin seedlings (Table 2). All the *P. irregulare* isolates were pathogenic toward lupin, causing a significant reduction in

seedling survival and length, with the exception of isolate R8304C (PY4), which only caused a significant reduction in seedling length (Table 2). The *P. irregulare* isolates differed in virulence toward lupin; for example, isolate R8308F (PY2) caused significantly more seedling damping-off than most of the other *P. irregulare* isolates, with the exception of R8160L (Py8) (Table 2). Only one (R8201C) of the three *P. mamillatum* isolates was pathogenic and caused a significant reduction in seedling survival and length compared with the uninoculated control, whereas the other two isolates did not (Table 2). All the *P. myriotylum* isolates were pathogenic, because all the isolates caused a significant reduction in seedling length and survival, with the exception of isolate R8166K1 that was less virulent and only caused a significant reduction in seedling length. The *P. pyrilobum* isolates were all pathogenic, causing a significant reduction in seedling survival and length. One of the *P. pyrilobum* isolates (R8303J) was highly virulent, causing significantly more seedling damping-off than all of the other oomycete isolates (Table 2).

The pathogenicity assays on oat revealed that some isolates in *P. irregulare*, *P. myriotylum*, and *P. pyrilobum* were pathogenic, causing significantly less seedling survival (Table 2) and a significant reduction in seedling length (Table 2) when compared with the uninoculated control. Most isolates were not highly virulent and caused less than 30% damping-off, with the exception of some *P. myriotylum* isolates that caused more than 50% damping-off. All *Phytophthora cinnamomi*, *Pythium acanthicum*, and *P. mamillatum* isolates were nonpathogenic and did not cause a significant reduction in seedling survival or length (Table 2). Similar to what was found on lupin, isolates within species differed in their pathogenicity. Four of the *P. irregulare* isolates (R8129Q, R8304C, R8307N, and R8161C) were nonpathogenic and did not cause a significant reduction in seedling length or survival. The remaining *P. irregulare* isolates were pathogenic, causing a significant reduction in seedling length, although only a few isolates (R8162D, R8167A, R8171D, and R8160A) were able to cause a significant reduction in seedling survival. Only three (R8169S, R8197F, and R8204F) of the six *P. myriotylum* isolates were pathogenic, causing a significant reduction in seedling survival (Table 2) and length (except isolate R8169S) compared with the uninoculated control (Table 2). All the *P. pyrilobum* isolates were pathogenic, causing a significant reduction in seedling survival and length (Table 2).

Evaluating the potential of nonpathogenic *Pythium* spp. to suppress damping-off caused by pathogenic oomycete species. In co-inoculation pathogenicity assays, there was not enough evidence ($P > 0.05$) against homogeneity of variances of the two repeat trials for seedling survival and length; thus, the data of the two repeat trials were combined. There was not enough evidence for significant interaction ($P \geq 0.05$) between the trial and other factors; therefore, a combined analysis with six replicates (blocks with trials) was performed. The analyses of variance on the percent survival and seedling length showed a significant treatment-crop interaction ($P < 0.0001$) (Supplementary Table S2). Therefore, it was appropriate to calculate the interaction *t*-LSD to compare the interaction means within rows and within columns (Table 3).

The co-inoculation of pathogenic (*Phytophthora cinnamomi*, *Pythium irregulare*, *P. mamillatum*, *P. myriotylum*, and *P. pyrilobum*) species and nonpathogenic *Pythium* spp. (*P. acanthicum*, *P. cederbergense*, and *Pythium* RB II) showed that nonpathogenic species were effective in suppressing disease only in the less susceptible crops lupin and oat but not on rooibos (Table 3). On rooibos, the presence of nonpathogenic *Pythium* spp. along with pathogenic oomycete species still resulted in 100% damping-off of seedlings (Table 3). On lupin, when several pathogenic oomycete species were present (M1), nonpathogenic species resulted in a significant increase in seedling length but no significant effect was seen for a reduction in damping-off (Table 3). In contrast, when only *P. irregulare* was inoculated on lupin, the nonpathogenic *Pythium* spp. (M4) were able to suppress disease and caused a significant increase in seedling survival and length compared with when only *P. irregulare* (M6) was present (Table 3). It was unex-

pected to find that, when nonpathogenic *Pythium* spp. were combined with *Phytophthora cinnamomi* (M5), a significant reduction in seedling survival was observed compared with when only *P. cinnamomi* (M7) was present. On oat, co-inoculation of nonpatho-

genic *Pythium* spp. with pathogenic oomycete species (M1), resulted in a significantly higher survival rate and length of oat seedlings compared with when only pathogenic species were present (M2) (Table 3). It should be noted, however, that the *Pythium*

Fig. 3. Phylogeny of *Pythium* collected from rooibos and *Pythium* clade F (34,63) based on the internal transcribed spacer region of nuclear ribosomal DNA. The tree is one of 920 equally parsimonious trees of a heuristic search. Rooibos isolates are indicated by a + symbol. CBS reference isolates are indicated in bold. Numbers in the tree represent parsimony bootstrap support values followed by posterior probabilities (in parentheses). Bootstrap support of 100% and a probability value of 1.00 are indicated by a star symbol (*). Bootstrap support less than 60% and probability values less than 0.70 are not indicated. Tree length (TL) = 923; consistency index (CI) = 0.691; retention index (RI) = 0.854; rescaled consistency index (RC) = 0.590; homoplasy index (HI) = 0.309.

mamillatum and *Phytophthora cinnamomi* isolates in the pathogenic species mix were nonpathogenic toward oat (Tables 2 and 3). On oat, the *Pythium irregulare* isolate by itself only caused a reduction in seedling length but, when nonpathogenic species were added, this reduction in length was significantly reduced (Table 3).

Discussion

Several oomycete species were found associated with rooibos grown in nurseries and in native rooibos sites in the Western Cape Province of South Africa. Most of the oomycete species consisted of *Pythium* spp., including *Pythium acanthicum*, *P. irregulare*, *P. mamillatum*, *P. myriotylum*, *P. pyrilobum*, *P. cederbergense*, and *Pythium* RB II, but one *Phytophthora* sp., *Phytophthora cinnamomi*, was also identified. The species composition in the rooibos nurseries and the native sites was similar with regards to the predominance by *Pythium irregulare* and the occurrence of *P. mamillatum*. *P. acanthicum*, *P. myriotylum*, and *Pythium* RB II were only identified in nurseries. *Phytophthora cinnamomi* and *Pythium pyrilobum* were only detected in the native sites. The detection of *Phytophthora cinnamomi* in a native site might be due to this species being the most virulent and widespread pathogen of the indigenous family Proteaceae (13,70) that is native to the region where rooibos

naturally occurs. The oomycete species identified from rooibos in the current study do not include any new species reports from South Africa (9,44); however, this is the first report of these species being associated with rooibos.

In several but not all rooibos locations, more than one oomycete species was associated with rooibos. In the native rooibos sites, up to three different species were identified from the same site, with most sites containing only two species. Two of the rooibos nurseries contained four species. In both of these nurseries, *Pythium myriotylum* dominated the populations. The prevalence of one *Pythium* sp. and several species being associated with a specific host is not uncommon, and has been reported for several crops, including wheat (*Triticum aestivum* L.), grapevine (*Vitis vinifera* L.), apple, and carrot (*Daucus carota* L.). On these crops, the species composition was also found to differ with location (40,49,64,65). Biological traits such as pathogenicity, optimum temperature for mycelial growth, and saprophytic survival of inoculum could explain the fluctuations in *Pythium* spp. composition (65). Several abiotic soil properties can also affect *Pythium* community composition in soil, including pH, calcium, magnesium, and field capacity (4).

P. irregulare, a genetically diverse species, is an important pathogen of rooibos due to its prevalence and high virulence toward

Table 2. Percent survival and seedling length of lupin, oat, and rooibos seedlings grown in soil inoculated with different *Pythium* isolates, *Phytophthora cinnamomi*, and an uninoculated control

Species, isolate ^z	Survival (%) ^x			Length (mm) ^y		
	Lupin	Oat	Rooibos	Lupin	Oat	Rooibos
Control	74.1	94.6	94.0	111.4	136.2	32.8
<i>P. cinnamomi</i>						
R8303A (PY9)	38.3	94.3	0.0	71.6	151.0	—
<i>Pythium acanthicum</i>						
R8162U (PY10)	63.3	93.3	92.0	103.5	141.3	23.5
R8206Q (PY10)	70.8	95.3	89.7	109.0	140.5	28.5
<i>P. irregulare</i>						
R8282W1 (PY1)	55.0	88.0	0.0	84.9	117.6	—
R8294C (PY1)	60.0	86.0	0.0	70.7	114.2	—
R8162D (PY2)	52.5	82.0	0.0	88.1	112.6	—
R8167A (PY2)	60.0	80.0	0.0	76.9	116.5	—
R8171D (PY2)	51.6	79.0	0.0	61.9	115.1	—
R8308F (PY2)	35.8	88.7	0.0	65.2	114.0	—
R8129Q (PY4)	59.1	89.7	0.0	92.2	131.4	—
R8304C (PY4)	65.8	89.7	0.0	88.0	125.5	—
R8307N (PY4)	60.0	92.7	0.0	92.8	125.5	—
R8161C (PY4)	62.5	91.3	0.0	96.9	129.2	—
R8244H (PY4)	59.1	91.0	0.0	97.6	121.5	—
R8168I (PY11)	58.3	84.0	0.0	86.3	106.5	—
R8177A (PY5)	61.6	92.7	0.0	80.8	119.4	—
R8292AE (PY5)	55.8	86.0	0.0	86.4	112.5	—
R8160A (PY8)	57.5	79.0	0.0	72.0	109.0	—
R8160L (PY8)	46.6	84.0	0.0	62.1	104.0	—
<i>P. mamillatum</i>						
R8201C (PY6)	45.0	91.0	0.0	88.6	126.0	—
R8296C (PY6)	65.0	88.0	0.0	100.5	124.0	—
R8302D (PY6)	75.0	91.7	0.0	106.5	125.2	—
<i>P. myriotylum</i>						
R8128T (PY3)	51.6	89.0	0.0	88.7	131.6	—
R8130U (PY3)	51.6	84.3	0.0	76.4	126.1	—
R8166K1 (PY3)	63.3	93.0	0.0	96.5	130.0	—
R8169S (PY3)	41.6	69.6	0.0	67.3	124.5	—
R8197F (PY3)	35.0	48.3	0.0	52.4	104.5	—
R8204F (PY3)	48.3	57.3	0.0	59.8	101.8	—
<i>P. pyrilobum</i>						
R8298A (PY7)	55.8	77.6	0.0	79.0	106.9	—
R8302F (PY7)	57.5	67.6	0.0	89.1	111.0	—
R8303D (PY7)	42.5	75.3	0.0	80.6	111.8	—
R8303J (PY7)	26.6	66.0	0.0	46.5	108.6	—

^x Seedling survival was evaluated 2 weeks after planting seed. Values are the mean of three replicates, and data that were pooled over two trials. Least significant difference (LSD; $P = 0.05$) = 11.38.

^y Length of lupin and oat seedlings was determined 2 weeks after sowing seed, and after 4 weeks for rooibos. Values are the mean of three replicates, and data that were pooled over two trials; — indicates no seedlings survived. LSD ($P = 0.05$) = 12.88.

^z Internal transcribed spacer region polymerase chain reaction restriction fragment length (ITS-PCR-RFLP) group (PY1 to PY11) to which each isolate belonged to are indicated in parentheses.

rooibos. All of the nurseries contained *P. irregulare* and, in most of the nurseries (58%), only *P. irregulare* was identified. Six different PCR-RFLP groups were identified for the nursery isolates. The same PCR-RFLP groups also occurred in the native sites, where *P. irregulare* was also prevalent, occurring in 46% of the sites. Phylogenetic analyses of representatives of the different *P. irregulare* PCR-RFLP groups showed that isolates from rooibos were genetically diverse at the ITS level, as previously reported (3,19,34,39). The rooibos isolates fit into two subclades within the *P. irregulare* species complex, which include one novel subclade when compared with the subclades identified by Spies et al. (63). BLAST analyses would suggest that some of the rooibos *P. irregulare* isolates could have been identified as *P. cryptoirregulare* but the validity of this species has been questioned (63). Therefore, all the rooibos isolates were considered to be *P. irregulare*. The diversity in *P. irregulare* was also evident in pathogenicity studies toward oat and lupin but there was no correlation between the observed genetic variation and variation in pathogenicity. On oat, not all of the *P. irregulare* isolates were pathogenic whereas, on lupin, some isolates differed in virulence. Spies et al. (64) also identified nonpathogenic *P. irregulare* isolates among pathogenic isolates on grapevine. Variation in virulence among *P. irregulare* isolates has also been reported on barrel medic (*Medicago truncatula* Gaertn.), subclover (*Trifolium subterraneum* L.), and wheat (22). Variation in pathogenicity and virulence among *P. myriotylum* isolates, as has been identified in the current study, have also been reported on cocoyam (*Xanthosoma sagittifolium* (L.) Schott) (50), tomato (*Solanum lycopersicum* L.), rye (*Secale cereal* L.), oat, wheat, sorghum (*Sorghum bicolor* (L.) Moench), peanut (*Arachis hypogaea* L.), bean (*Phaseolus vulgaris* L.), tobacco (*Nicotiana tabacum* L.), and soybean (*Glycine max* (L.) Merr.) (43).

Several of the rooibos-associated oomycete species were shown to be highly virulent toward rooibos and less virulent on the two rotation crops lupin and oat. Almost all oomycete species associated with rooibos, except *Pythium acanthicum* and the previously characterized *P. cederbergense* and *Pythium* RB II, were highly virulent toward rooibos, causing 100% damping-off. All the species that were pathogenic on rooibos were also pathogenic on lupin but, on oat, only *P. irregulare*, *P. myriotylum*, and *P. pyriformis* were pathogenic. *P. irregulare* is known as a highly virulent species with a very wide host range (5,9,31,38,48,69) and has been reported as a pathogen of lupin (66,68) but not of oat. *Phytophthora cinnamomi* has also been reported as a pathogen of lupin (61) and *Pythium myriotylum* as a pathogen of oat (43). Thus, this is the first report of *P. mamillatum*, *P. pyriformis*, and *P. myriotylum* as pathogens of lupin and *P. irregulare* and *P. pyriformis* as pathogens of oat. Although *P. mamillatum* and *P. myriotylum* have not been reported as pathogens of lupin, they have been reported as pathogens of other legume crops, including peanut, cowpea (*Vigna unguiculata* (L.) Walp.), soybean (pathogen: *P. myriotylum*), and kidney bean (*Phaseolus vulgaris* L.; pathogens: *Pythium mamillatum* and *P. myriotylum*) (38,52,56). *P. pyriformis* has not been reported as a pathogen of other legume crops. Although *P. irregulare* and *P. pyriformis* have not been reported as pathogens of oat, *P. irregulare* has been reported as a pathogen of wheat and *P. pyriformis* as a pathogen of rice (it only reduced root growth but did not reduce shoot growth or seedling survival; 5,8). Although *P. acanthicum* has not been reported as a pathogen of oat and lupin or other legume and grain crops, it has been reported as a pathogen of tomato (68) and watermelon (68). Considering the wide host range and diversity of oomycete species associated with rooibos, it might be difficult to find a rotation crop that is a nonhost and that can significantly reduce oomycete inoculum levels.

Nonpathogenic *Pythium* spp. (*P. acanthicum*, *P. cederbergense*, and *Pythium* RB II) showed potential for suppressing damping-off caused by *P. irregulare* or a combination of oomycetes (*Phytophthora cinnamomi*, *Pythium irregulare*, *P. mamillatum*, *P. myriotylum* and *P. pyriformis*) on lupin and oat but not on rooibos. On lupin, the nonpathogenic *Pythium* spp. caused a significant reduction in symptoms caused by *P. irregulare* and by a combination of pathogenic oomycetes (*Phytophthora cinnamomi*, *Pythium irregulare*, *P. mamillatum*, *P. myriotylum*, and *P. pyriformis*). It was unexpected that the combination of *Phytophthora cinnamomi* and nonpathogenic *Pythium* spp. resulted in a significant increase in damping-off on lupin, compared with when only *P. cinnamomi* was present. This suggests that there might be a synergistic interaction between *P. cinnamomi* and the nonpathogenic *Pythium* spp. in causing disease on lupin. On oat, the nonpathogenic *Pythium* spp. were able to significantly reduce disease incited by the combination of oomycete species and when only *Pythium irregulare* was present. However, it should be noted that, in the mixture of isolates that was used, the only isolates that were pathogenic toward oat were *P. irregulare*, *P. myriotylum*, and *P. pyriformis*. However, the control achieved with all of the aforementioned nonpathogenic species was marginal because some only increased seedling length but did not decrease damping-off. Furthermore, where damping-off was suppressed, the level of control was not comparable with the uninoculated control, as growers often expect when applying a management treatment. Improved disease suppression might be obtained if more isolates of the nonpathogenic species are evaluated, because biological control isolates are known to vary in efficacy, with some isolates being superior in suppressing specific diseases on specific crops. In the future, it will also be important to evaluate the biocontrol efficacy using naturally infested pathogen field soils and artificial inoculation of the nonpathogens, because the current study evaluated the biocontrol potential using artificial inoculation of pathogens into a pasteurized potting mix. This will also determine whether the nonpathogens will be able to establish

lata (L.) Walp.), soybean (pathogen: *P. myriotylum*), and kidney bean (*Phaseolus vulgaris* L.; pathogens: *Pythium mamillatum* and *P. myriotylum*) (38,52,56). *P. pyriformis* has not been reported as a pathogen of other legume crops. Although *P. irregulare* and *P. pyriformis* have not been reported as pathogens of oat, *P. irregulare* has been reported as a pathogen of wheat and *P. pyriformis* as a pathogen of rice (it only reduced root growth but did not reduce shoot growth or seedling survival; 5,8). Although *P. acanthicum* has not been reported as a pathogen of oat and lupin or other legume and grain crops, it has been reported as a pathogen of tomato (68) and watermelon (68). Considering the wide host range and diversity of oomycete species associated with rooibos, it might be difficult to find a rotation crop that is a nonhost and that can significantly reduce oomycete inoculum levels.

Table 3. Percent survival and length of lupin, oat and rooibos seedlings grown in soil co-inoculated with different combinations of *Pythium* spp. (pathogenic and nonpathogenic) and *Phytophthora cinnamomi*

Treatment	Survival (%) ^w			Length (mm) ^x		
	Lupin	Oat	Rooibos	Lupin	Oat	Rooibos
Control	69.2 ef	93.0 ab	96.7 a	120.4 ef	146.5 ab	33.4 i
M1 (pathogenic oomycete spp. and nonpathogenic <i>Pythium</i> spp.) ^{y,z}	45.0 hi	74.7 def	0.0 k	90.7 g	133.1 cd	–
M2 (pathogenic oomycete spp.) ^y	40.8 i	53.7 gh	0.0 k	65.0 h	101.6 g	–
M3 (nonpathogenic spp.) ^z	66.7 f	90.3 ab	93.0 ab	125.9 de	144.1 bc	33.1 i
M4 (<i>Pythium irregulare</i> and nonpathogenic <i>Pythium</i> spp.) ^z	55.8 g	79.0 cde	0.0 k	92.7 g	130.5 de	–
M5 (<i>Phytophthora cinnamomi</i> and nonpathogenic <i>Pythium</i> spp.) ^z	19.2 j	85.0 bcd	0.0 k	76.0 h	157.0 a	–
M6 (<i>Pythium irregulare</i>)	35.8 i	88.7 abc	0.0 k	67.8 h	114.0 f	–
M7 (<i>Phytophthora cinnamomi</i>)	38.3 i	94.3 ab	0.0 k	71.1 h	151.0 ab	–

^w Means followed by the same letter do not differ significantly at *P* = 0.05. Seedling survival was evaluated 2 weeks after planting seed. Values are the mean of three replicates, and data that were pooled over two trials. Least significant difference (LSD; *P* = 0.05) = 10.64.

^x Means followed by the same letter do not differ significantly at *P* = 0.05. The length of lupin and oat seedlings was determined 2 weeks after sowing seed, and after 4 weeks for rooibos. Values are the mean of three replicates, and data that were pooled over two trials; – indicates no seedlings survived. LSD (*P* = 0.05) = 11.90.

^y The same pathogenic oomycete species were included in treatment M1 and M2 and consisted of one isolate each of *Phytophthora cinnamomi*, *Pythium irregulare*, *P. mamillatum*, *P. myriotylum*, and *P. pyriformis*.

^z Nonpathogenic species consisted of one isolate each of *P. acanthicum*, *Pythium* RB II, and *P. cederbergense*.

in a natural field soil where a consortium of native microbes are already present.

The suppression of *Pythium* diseases by species within the same genus has previously been reported by a few other studies. *P. acanthicum* was able to reduce damping-off caused by *P. ultimum* on cucumber (1) and damping-off caused by *P. irregulare* and *P. mamillatum* on sugar beet (55). *P. periplocum* and *P. oligandrum* have been reported to reduced damping-off of sugar beet caused by *P. ultimum* (55). Ribeiro and Butler (55) also showed that *P. irregulare* and *P. mamillatum* were highly susceptible to *P. oligandrum* and *P. periplocum*, while *P. myriotylum* was highly susceptible to *P. periplocum*. *Phytophthora cinnamomi* was only marginally suppressed by *Pythium acanthicum* and *P. periplocum* (55). The three modes of parasitism implicated in disease suppression by mycoparasitic *Pythium* spp. include lysis, cytoplasmic coagulation, and penetration of the host at the point of contact (29,55).

The current study showed that *P. irregulare* is a prominent and virulent pathogen of rooibos, along with several other pathogenic species that were less frequently found. Most of the pathogenic rooibos species were also pathogenic toward two nursery rotation crops, lupin and oat. On these crops, there were fewer species and isolates that were pathogenic toward oat, and the species were also less virulent on oat and lupin than on rooibos. The reduced number of species and isolates pathogenic toward oat compared with lupin could explain field observations that lupin as a pre-crop significantly increased damping-off of rooibos whereas oat did not (*unpublished data*). The biocontrol effect of oat has previously been demonstrated in a study by Deacon and Mitchell (12) that showed that oat roots attract and lyse zoospores of several *Pythium* spp. (*P. aphanidermatum* (Edson) Fitzp., *P. arrhenomanes* Drechsler, *P. graminicola* Subraman., *P. intermedium* de Bary, *P. ultimum* var. *sporangiiferum* Drechsler), and *Phytophthora cinnamomi*. Oat roots were also found to inhibit oospore formation and germination, due to the release of fungitoxic compounds from the roots (12). The effective use of oat as a rotation crop will have to be evaluated further in glasshouse and field trials. It will also be important to determine whether oat can result in an increase in non-pathogenic oomycete species that could help suppress pathogenic species on oat and, perhaps, on rooibos. Ultimately this, along with disease incidence, will determine whether oat will form an important part of an integrated management strategy of rooibos damping-off caused by oomycetes. The use of nonpathogenic *Pythium* spp. as biocontrol agents in rooibos nurseries as a standalone treatment did not show promise, because it did not suppress damping-off on rooibos. It is also of concern that, on lupin, the nonpathogenic species enhanced the virulence of *P. cinnamomi*.

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