PYTHIUM SPECIES ASSOCIATED WITH ROOIBOS, AND THE INFLUENCE OF MANAGEMENT PRACTICES ON DISEASE DEVELOPMENT

By

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DECLARATION

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SUMMARY

Damping-off of rooibos (*Aspalathus linearis*), which is an important indigenous crop in South Africa, causes serious losses in rooibos nurseries and is caused by a complex of pathogens of which oomycetes, mainly *Pythium,* are an important component. The management of damping-off in organic rooibos nurseries is problematic, since phenylamide fungicides may not be used. Therefore, alternative management strategies such as rotation crops, compost and biological control agents, must be investigated. The management of damping-off requires knowledge, which currently is lacking, of the *Pythium* species involved, and their pathogenicity towards rooibos and two nursery rotation crops (lupin and oats). *Pythium* species identification can be difficult since the genus is complex and consists of more than 120 species. Species identification is, however, greatly facilitated by analyses of the internal transcribed spacer (ITS) regions. These regions, have also been used to divide the genus into 11 phylogenetic clades (A to K), with some clades, such as clade G, still being poorly characterised.

The first aim of the study was to characterize 12 *Pythium* clade G isolates that were obtained from damped-off rooibos seedlings, along with six known clade G species. Subsequently, oligonucleotides were designed for differentiating two rooibos associated groups that may represent new taxons, for future use in DNA macro-array analyses. Phylogenetic analyses of the ITS region and a combined phylogeny of four gene regions (ITS, β-tubulin and, *COX1* and *COX2* [cytochrome c oxidase subunits I and II]) identified five sub-clades within *Pythium* clade G. The rooibos isolates formed two groups, Rooibos group I (RB I) and II (RB II) that clustered into two groups within sub-clade 1 with good support (64%-89% bootstrap, 1.00 probability). The *Pythium* RB I isolates had *P. iwayamai* as its nearest neighbour, and may represent a new species. The *Pythium* RB II isolates had *P. canariense* and *P. violae* as their closest relatives and may, along with other isolates contained in the RB II sub-clade, represent several new species. Morphological analyses of the rooibos isolates were inconclusive, since the isolates all contained similar morphological

characteristics that did not correspond to the description of known *Pythium* species. The *Pythium* RB I and II isolates were all non-pathogenic toward rooibos, lupin and oats seedlings. For each of the two rooibos groups, one newly developed oligonucleotide was able to differentiate the isolates from clade G reference isolates using DNA macro-array analyses.

The second aim of the study was to determine the oomycetes species associated with rooibos in nurseries and in a native rooibos site, and their pathogenicity towards rooibos and two nursery rotation crops (lupin and oats). Since some isolates were shown to be non-pathogenic, another aim was to determine whether these isolates, along with the previously characterised non-pathogenic *Pythium* RB I and RB II isolates, could suppress pathogenic oomycetes. Characterisation of isolates from 19 nurseries and one native rooibos site revealed the presence of five *Pythium* species (*P. acanthicum, P. irregulare, P. mamillatum, P. myriotylum,* and *P. pyrilobum*) and *Phytophthora cinnamomi.* In nurseries, *P. irregulare* was the most common species (81%) followed by *P. myriotylum* (14%). Similarly, *P. irregulare* was also the most prevalent species (57%) in native rooibos, but *P. pyrilobum* (26%) was second most prevalent. Pathogenicity studies on rooibos showed that all species, except *P. acanthicum,* were highly virulent causing 100% damping-off. On lupin, *P. acanthicum* was also the only non-pathogenic species, with the other species being less virulent on lupin than on rooibos. Only *P. irregulare, P. myriotylum,* and *P. pyrilobum* were pathogenic towards oats, and were also less virulent on oats than on rooibos. On lupin and oats, not all off the isolates from a specific species was pathogenic. Non-pathogenic *Pythium* species (*P. acanthicum*, *Pythium* RB I and II) was only effective at suppressing disease on the less susceptible crops of lupin and oats, but not on rooibos.

The third aim of the study was to investigate the management of rooibos damping-off using two composts (A and B), and composts combined with non-pathogenic *Pythium* species. Evaluation of the suppression by composts of *Ph. cinnamomi* and 29 *Pythium* isolates, which represented the four pathogenic *Pythium* rooibos species, showed that both composts were able to suppress some, but not all of the pathogenic *Pythium* isolates. Both composts were very effective at, and the highest percentage control was achieved, with suppression of *Ph. cinnamomi.* Most isolates of *P. mamillatum* and *P. pyrilobum* were suppressed by composts, whereas most *P. irregulare* (> 62%) and *P. myriotylum* (>50%) isolates were not suppressed. Non-pathogenic *Pythium* species combined with either of the two composts were able to significantly reduce damping-off caused by *P. irregulare* or a combination of pathogenic species (*P. irregulare, P. mamillatum, P. myriotylum, P.*

pyrilobum, and *Ph. cinnamomi*), compared to than when only the pathogens were present. In the absence of non-pathogenic species, neither of the composts was able to suppress the aforementioned pathogenic isolates.

This study has improved our knowledge of the oomycete species that are involved in rooibos damping-off, and has identified possible management strategies for use in organic nurseries. Several oomycete species are involved in causing damping-off and their differential virulence, and responses to being suppressed by composts, will require the use of integrated management strategies. Management strategies that showed promise include the combined use of compost and non-pathogenic *Pythium* taxons. The use of oats, which is susceptible to fewer oomycete isolates than rooibos, could also be valuable as a rotation crop. Altogether, knowledge obtained in this study can be used to (i) optimize integrated management strategies for organic nurseries, (ii) elucidate the mechanisms involved in disease suppression and (ii) develop molecular techniques, such as DNA macro-arrays and quantitative PCR (qPCR) for the rapid assessment of the species involved, and the quantification of inoculum in nursery soils.

PYTHIUM SPESIES WAT MET ROOIBOS GEASSOSIEER WORD, EN DIE INVLOED VAN BESTUURSPRAKTYKE OP SIEKTE-ONTWIKKELING

OPSOMMING

Omvalsiekte van rooibos (*Aspalathus linearis*), wat 'n belangrike inheemse gewas in Suid-Afrika is, veroorsaak ernstige verliese in rooiboskwekerye, en word deur 'n kompleks van patogene veroorsaak, waarvan oömysete, hoofsaaklik *Pythium,* 'n belangrike komponent is. Die bestuur van omvalsiekte in organiese rooiboskwekerye is problematies, aangesien fenielamied fungisiedes nie gebruik mag word nie. Alternatiewe bestuurstrategieë, soos rotasie-gewasse, kompos en biologiese beheer-agente, moet dus ondersoek word. Die bestuur van omvalsiekte vereis kennis, wat tans ontbreek, naamlik die *Pythium* spesies wat betrokke is, hul patogenisiteit teenoor rooibos, en twee kwekery rotasie-gewasse (lupiene en hawer). *Pythium* spesie-identifikasie kan moeilik wees aangesien die genus kompleks is en uit meer as 120 spesies bestaan. Spesie-identifikasie word egter grootliks vergemaklik deur analise van die interne getranskribeerde spasieerder (ITS) areas. Hierdie areas is ook gebruik om die genus in 11 filogenetiese "clades" (A tot K) te verdeel, met sommige "clades", soos "clade" G, wat steeds swak gekarakteriseer is.

Die eerste doelwit van die studie was om 12 *Pythium* "clade" G isolate te karakteriseer, wat vanaf omvalsiekte rooibossaailinge verkry is, tesame met ses bekende "clade" G spesies. Gevolglik is oligonukleotiede ontwerp ten einde twee rooibos-geassosieerde groepe, wat nuwe taksons kan verteenwoordig, te onderskei, en vir toekomstige gebruik in DNS makro-"array" analise. Filogenetiese analise van die ITS area en 'n gekombineerde filogenie van vier geen-areas (ITS, β-tubulien en, *COX1* en *COX2* [sitokroom c oksidase sub-eenhede I en II]) het vyf sub-"clades" binne *Pythium* "clade" G geïdentifiseer. Die rooibos isolate het twee groepe gevorm, Rooibos groep I (RB I) en II (RB II) wat twee groepe binne sub-"clade" 1 gevorm het, met goeie ondersteuning (64%-89% "bootstrap", 1.00 waarskynlikheid). Die *Pythium* RB I isolate het *P. iwayamai* as sy naaste verwant, en mag 'n nuwe spesie verteenwoordig. Die *Pythium* RB II isolate het *P. canariense* en *P. violae* as hul naaste verwante en mag, tesame met ander isolate wat in die RB II sub-"clade" ingesluit word, verskeie nuwe spesies verteenwoordig. Morfologiese analise van die rooibos-

isolate was onbeslis, aangesien die isolate almal soortgelyke morfologiese kenmerke bevat het, wat nie met die beskrywing van bekende *Pythium* spesies ooreengestem het nie. Die *Pythium* RB I en II isolate was almal nie-patogenies teenoor rooibos-, lupien- en hawersaailinge. Vir elk van die twee rooibosgroepe, was een nuut-ontwikkelde oligonukleotied in staat om die isolate van "clade" G verwysingsisolate te differensieer, deur die gebruik van DNS makro-"array" analise.

Die tweede doelwit van die studie was om die oömysete spesies wat met rooibos in kwekerye en in 'n inheemse rooibos-area geassosieer word, te bepaal, en hul patogenisiteit teenoor rooibos en twee kwekery rotasie-gewasse (lupien en hawer). Aangesien van die isolate nie-patogenies was, was 'n ander doelwit om te bepaal of hierdie isolate, tesame met die voorheen gekarakteriseerde nie-patogeniese *Pythium* RB I en RB II isolate, patogeniese oömysete kan onderdruk. Karakterisering van isolate van 19 kwekerye en een inheemse rooibos-area, het op die teenwoordigheid van vyf *Pythium* spesies (*P. acanthicum, P. irregulare, P. mamillatum, P. myriotylum,* en *P. pyrilobum*) en *Phytophthora cinnamomi* gedui. *P. irregulare* was die mees algemene spesie (81%) in kwekerye, gevolg deur *P. myriotylum* (14%). Soortgelyk was *P. irregulare* ook die mees algemene spesie (57%) in inheemse rooibos, maar *P. pyrilobum* (26%) was tweede mees algemeen. Patogenisiteitstudies op rooibos het getoon dat alle spesies, behalwe *P. acanthicum,* hoogs virulent was en 100% omvalsiekte veroorsaak het. Op lupien was *P. acanthicum* ook die enigste nie-patogeniese spesie, terwyl die ander spesies minder virulent op lupien as op rooibos was. Slegs *P. irregulare, P. myriotylum* en *P. pyrilobum* was patogenies teenoor hawer, en was ook minder virulent op hawer as op rooibos. Op lupien en hawer was nie alle isolate van 'n spesifieke spesie patogenies nie. Nie-patogeniese *Pythium* spesies (*P. acanthicum*, *Pythium* RB I en II) was slegs effektief om siekte op die minder vatbare gewasse, lupien en hawer, te onderdruk, maar nie op rooibos nie.

Die derde doelwit van die studie was om die bestuur van rooibos omvalsiekte te ondersoek, deur die gebruik van twee tipes kompos (A en B), en kompos gekombineer met nie-patogeniese *Pythium* spesies. Evaluasie van die onderdrukking deur kompos van *Ph. cinnamomi* en 29 *Pythium* isolate, wat die vier patogeniese *Pythium* rooibosspesies verteenwoordig het, het getoon dat beide tipes kompos in staat was om sommige, maar nie al die patogeniese *Pythium* isolate, te onderdruk nie. Beide tipes kompos was baie effektief, en die hoogste persentasie beheer was met die onderdrukking van *Ph. cinnamomi* verkry. Meeste isolate van *P. mamillatum* en *P. pyrilobum* is deur kompos onderdruk, terwyl meeste

P. irregulare (> 62%) en *P. myriotylum* (>50%) isolate nie onderdruk is nie. Nie-patogeniese *Pythium* spesies, in kombinasie met enige van die twee tipes kompos, was in staat om betekenisvol omvalsiekte veroorsaak deur *P. irregulare*, of in 'n kombinasie met patogeniese spesies (*P. irregulare, P. mamillatum, P. myriotylum, P. pyrilobum*, en *Ph. cinnamomi*), te verminder, in vergelyking met wanneer slegs die patogene aanwesig was. In die afwesigheid van nie-patogeniese spesies, was nie een van die tipes kompos in staat om die voorafgenoemde patogeniese isolate te onderdruk nie.

Hierdie studie het ons kennis rakende die oömysete spesies betrokke in rooibos omvalsiekte verbeter, en het moontlike bestuurstrategieë geïdentifiseer wat in organiese kwekerye gebruik kan word. Verskeie oömysete spesies is betrokke in die oorsaak van omvalsiekte, en hul verskille in virulensie, en reaksies op onderdrukking deur kompos, sal die gebruik van geïntegreerde bestuurstrategieë vereis. Bestuurstrategieë wat belofte toon, sluit die gekombineerde gebruik van kompos en nie-patogeniese *Pythium* taksons in. Die gebruik van hawer, wat vir minder oömysete isolate as rooibos vatbaar is, kan ook waardevol as 'n rotasie-gewas wees. Tesame, kan kennis wat in die studie opgedoen is gebruik word om (i) geïntegreerde bestuurstrategieë vir organiese kwekerye te optimaliseer, (ii) die meganismes betrokke in siekte-onderdrukking te bepaal, en (iii) molekulêre tegnieke, soos DNS makro-"arrays" en kwantitatiewe PKR (qPKR) te ontwikkel vir die vinnige bepaling van die spesies betrokke, en die kwantifisering van inokulum in kwekery-gronde.

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CONTENTS

1. CHARACETRISATION AND MANAGEMENT OF *PYTHIUM* IN ROOIBOS SEEDLING PRODUCTION

INTRODUCTION

Rooibos (*Aspalathus linearis* (N.L. Burm.) R. Dahlgr.) is grown commercially in the Western Cape Province in South Africa, mainly for the production of rooibos tea, but also for the manufacturing of various beauty products (Joubert *et al.*, 2008). The first 6 weeks of rooibos seedling growth is the most vulnerable stage of the crop in nurseries, since damping-off caused by *Pythium, Fusarium* and *Rhizoctonia* species results in significant losses. Of these soilborne plant pathogens, *Pythium,* is one of the most important.

The genus *Pythium* consists of more than 120 species, which have a range of pathogenic to beneficial interactions with plants (Alexopoulos *et al.*, 1996; Dick, 2001). Many *Pythium* species are important plant pathogens of several crops including deciduous fruit trees, vegetables, cereals and ornamentals (Van der Plaats-Niterink, 1981; Alexopoulos *et al.*, 1996). However, a substantial number of species are not pathogenic, but are saprophytic with some species even promoting plant growth and showing potential as biocontrol agents (Van der Plaats-Niterink, 1981; Martin & Loper, 1999). For example, *Pythium acanthicum* Drechsler*, P. oligandrum* Drechsler*,* and *P. periplocum* Drechsler have been used as biocontrol agents against various pathogenic *Pythium* species such as *P. irregulare* Buisman*, P. mamillatum* Meurs, *P. vexans* de Bary and *P. ultimum* Trow (Van der Plaats-Niterink, 1981; Martin & Hancock, 1987; Ali-Shtayeh & Saleh, 1999).

The identification, detection and quantification of *Pythium* species are important from a management point of view. *Pythium* species can be identified morphologically, but this is very difficult due to high intraspecific variations in their morphological characteristics (Lévesque & De Cock, 2004; McLeod *et al.*, 2009). Therefore, molecular techniques are very important in species identification. A valuable resource for the molecular identification of *Pythium* species consists of the work conducted by Lévesque and De Cock (2004), who constructed an internal transcribed spacer (ITS) phylogeny of 116 *Pythium* species. Subsequently, Tambong *et al.* (2006) developed a DNA macro-array for the detection of more than 100 *Pythium* species, which is a valuable tool for simultaneously identifying a

large number of species from environmental samples. In addition to DNA arrays, the polymerase chain reaction (PCR), either conventional or real-time PCR, has also been used to detect and quantify several pathogenic *Pythium* species (Cullen *et al.*, 2007; Le Floch *et al.*, 2007)

The management of *Pythium* diseases is difficult and requires an integrated approach, which can include cultural practices (water management, organic amendments and rotation crops), host resistance, fungicides and biological control agents (Boehm & Hoitink, 1992; Erwin & Ribeiro, 1996; Bates *et al.*, 2008). Fungicides, such as the phenylamides (e.g. metalaxyl), can provide good control of oomycetes and often yields more consistent control than some biological management strategies (Fry, 1982; Erwin & Ribeiro, 1996). The use of fungicides, however, is not allowed in organic production. Consequently, the integrated use of management practices such as compost, green manures, water management and rotation crops are important in these production systems. The disease suppressive potential of composts is well known (De Ceuster & Hoitink, 1999), although it has some limitations for suppressing soilborne diseases, such as a lack of consistency (Hoitink *et al.*, 1997). Therefore, investigations directed at identifying factors contributing to disease suppression and optimizing the use of compost for suppressing soilborne diseases are continuing (Veeken *et al.*, 2005; Termorshuizen *et al.*, 2006). Rotation crops have been useful for reducing diseases caused by some pathogens that produce long-lived survival structures (Huisman & Ashworth, 1976; Fry, 1982; Umaerus *et al.*, 1989), such as oospores produced by most *Pythium* species (Lévesque & De Cock, 2004). Green manures, which are cover crops that are incorporated into the soil after at least one season's growth, can also suppress *Pythium* populations, providing that long enough time is left after incorporation before planting. Planting of crops directly after incorporation of green manures can increase *Pythium* populations and disease (Grünwald *et al.,* 2000; Bonanomi *et al.*, 2010.

The aim of this review is to provide an overview of (i) the production and importance of rooibos in South Africa, (ii) the technologies that are used for the identification, detection and quantification of *Pythium* species and (iii) integrated management practices for managing *Pythium* damping-off, with an emphasis on the use of compost, green manures and crop rotation. Although *Phytophthora* is also an oomycete pathogen of rooibos, this pathogen will not be discussed in detail here, since it is not widely distributed in rooibos nurseries (Bahramisharif *et al.* 2011).

ROOIBOS

Rooibos only grows in the magnificent Cederberg Mountains, which is situated 200 km from Cape Town in South Africa. Rooibos is a broom-like member of the legume family of plants, and is used to make a herbal tea referred to as rooibos tea in South Africa. According to scientists, rooibos tea is healthy, since it is professed to assist nervous tension, allergies and digestive problems (Bramati *et al.*, 2002). The extracts have further been indicated as having the potential to prevent the progress of Human Immunodeficiency Virus (HIV) (Nakano *et al.*, 1997). Asplathin, a natural product that is isolated from rooibos leaves, (Koeppen & Roux, 1966) has potent antioxidant properties and radical scavenging activity (Gadow *et al.,* 1997) that can inhibit the proliferation of liver cancer cells (Snijman *et al.,* 2007). There is no scientific literature indicating that rooibos tea has any adverse effects on humans (McKay & Blumberg, 2007).

Commercial production of rooibos is limited to the heart of the Cederberg region, in the country village Clanwilliam. Rooibos production starts with the production of rooibos seed, singly in small pods that are formed from yellow, pea-shaped flowers that cover the plants in October. The rooibos seeds are released from the pods as soon as they are ripe, after which they are sieved from the soil surrounding the plants. Before planting the seeds, the seeds should be (i) scarred mechanically in order to improve the germination ability and (ii) treated with fungicides for protection against soilborne pathogens. The seeds are planted during February and March in nursery seed beds that are prepared and ploughed in the winter time. Rooibos seedlings are transplanted from seedbeds into commercial fields during July and August. Once the seedlings have been harvested in the nursery, lupin (*Lupinus angustifolius* L.) and oats (*Avena sativa* L.) are mainly used as rotation crops in the nurseries before the next seedling crop is produced. In commercial fields, the first crops are ready for harvest after 18 months, i.e. from January to April. The harvested green rooibos are cut into small pieces and left for the fermentation process, after irrigation has been applied. Subsequently, the rooibos material is spread on the yard to dry under the hot African sun. Finally, it is collected by vacuum machines and delivered to the factory (http://www.rooibosltd.co.za; Johan Brand, Rooibos Ltd., Clanwilliam, South Africa, personal communication).

THE GENUS *PYTHIUM* PRINGSHEIM

Taxonomy. The genus *Pythium* belongs to the phylum Oomycota, which although being fungus-like, have some significant differences in their biology when compared to true fungi. The oomycete cell wall is composed of cellulosic compounds and glucans, as opposed to true fungi that have cell walls that are mainly composed of chitin (Erwin & Ribeiro, 1996; Hardham, 2005). Another difference is the diploid thallus and completion of meiosis just before sexual reproduction in Oomycota, whereas true fungi are haploid for the most part of their life cycle (Erwin & Ribeiro, 1996; Hardham, 2005). The genus *Pythium* is placed within the class Oomycetes, order Peronosporales and family Pythiaceae. The genus is comprised of more than 120 described species (Dick, 1990), with several species still undergoing the identification process.

Life cycle. *Pythium* has a sexual and asexual life cycle that each has specific reproductive structures. The formation of oospores, which are the sexual spores, may require the presence of a corresponding compatibility type, in which case the isolate is said to be heterothallic. Alternatively, in homothallic species, oospores are formed in single culture in the absence of a corresponding compatibility type. Most *Pythium* species are homothallic. Oospores are hard, thick-walled structures that play an important role in survival, and they are also important for the generation of new genotypes (only heterothallic species). Some species also form hyphal swellings that are also an important overwintering structure (Van der Plaats-Niterink, 1981; Judelson, 2009). The asexual structures consist of sporangia and zoospores that are important for the rapid proliferation and dispersal of *Pythium* (Van der Plaats-Niterink, 1981). For some *Pythium* species, e.g. *P. heterothallicum* W. A. Campb. & F. F. Hendrix and *P. splendens* Braun, the sporangial form is unknown (Dick, 1990). Sporangia, which release motile zoospores, are produced from hyphal branches, or from germinating oospores (Martin & Loper, 1999; Hardham, 2007). Under certain conditions, oospores can germinate by only producing a germination tube without the production of a sporangium. Sporangia are formed and release zoospores under wet conditions, where the zoospores swim toward roots, encyst and infect the host. Infection of the host can also take place through germ tubes formed from oospores or hyphae (Van der Plaats-Niterink, 1981).

Ecology of *Pythium*. Water and soil temperatures are important abiotic factors in the interaction of *Pythium* with host plants. Free water plays an important role in the proliferation

of *Pythium* species (Martin & Loper, 1999; Broders *et al.*, 2009), since it allows motile zoospores to be attracted to, and swim towards germinating seeds or roots (Martin & Loper, 1999). This is due to the release of exudates from roots and seeds, which stimulate the germination of *Pythium* (Nelson, 1990). Soil temperature can differentially affect the disease severity incited by *Pythium* species, for example *P. aphanidermatum* (Edson) Fitzp. and *P. myriotylum* Drechsler are known to cause more damage in warmer areas, often at temperatures above 27˚C (Littrell & McCarter, 1970). In contrast, diseases caused by *P. irregulare* and *P. ultimum* can be more devastating at lower temperature ranges (15 to 25 °C), with *P. irregulare* causing damping-off of peas at temperatures as low as 5˚C (Thomson *et al.*, 1971; Pieczarka and Abawi, 1978; Ingram and Cook, 1990).

The ecology of *Pythium* in the spermosphere (area surrounding a germinating seed) is dynamic, since the spermosphere is a short-lived, rapidly changing, and microbiologically dynamic zone. The exudates released from seeds that begin to hydrate not only influence the behavior of *Pythium,* but also many other microbes that use the carbon released by seeds as their main energy source. Consequently, the behavior of *Pythium* in the spermosphere is strongly influenced by other microbes in the spermosphere.

The temporal responses of *Pythium* to seed exudates have been studied best in *P. ultimum* (Nelson, 2004). The germination of *P. ultimum* oospores starts with the thinning of the oospore wall (Ayers & Lumsden, 1975; Lumsden & Ayers, 1975; Johnson & Arroyo, 1983) that can be enhanced by oxygen, a pH above 6.5 (Johnson, 1988), soil moisture and temperatures at or above 25 °C (Lumsden & Ayers, 1975; Lifshitz & Hancock, 1984). This process can develop over a period of 15 days to 10 weeks, but once the wall is thinned, the oospore can germinate within 2 h (Lumsden & Ayers, 1975). Sporangia (zoosporangia and hyphal swellings) of *P. ultimum* are very responsive to seed exudates, and they start germinating within 1 h to 1.5 h after seed exudate exposure (Stanghellini & Hancock, 1971a, b; Nelson & Craft, 1989; Nelson & Hsu, 1994; McKellar & Nelson, 2003; Kageyama & Nelson, 2003). Subsequent germtube growth is fast being at least 300 µm/h (Stanghellini & Hancock, 1971b). The rapid sporangial germination response in *P. ultimum* is triggered by long-chain unsaturated fatty acids present in seed exudates (Ruttledge & Nelson, 1997), although the early literature indicated sugars and amino acids as being the trigger (Nelson, 1990). Seeds are rapidly colonized by *P. ultimum,* within 2 h to 4 h after seeds have been

planted, with all seeds being colonized 12 h to 24 h after planting (Stanghellini & Hancock, 1971a; Stasz & Harman, 1980; Hadar *et al.*, 1983; Lifshitz *et al.*, 1986; Nelson, 1988; Parke *et al.*, 1991; McKellar & Nelson, 2003). *Pythium ultimum* populations will start increasing around germinating seeds within 48 h of sowing (Nelson, 2004). In contrast to *P. ultimum*, oospores of *P. aphanidermatum* do not require a thinning of the oospore wall, and oospores can germinate within 1.5 h after exposure to seed exudates (Burr & Stanghellini, 1973; Stanghellini & Burr, 1973). Sporangia of *P. aphanidermatum* also germinate rapidly within 1.5 h in response to seed exudates. Most of the sporangia seem to germinate directly in the presence of seed exudates, and zoospores are only known to be released in the absence of seed exudates (Nelson, 2004).

Identification of *Pythium* species. The identification of *Pythium* species can be conducted using morphological and molecular methods. Several morphological keys have been published for the identification of *Pythium* species (Van der Plaats-Niterink, 1981; Dick, 1990). These keys use the presence, shape and size of sporangia, oogonia and hyphal swellings, the position and shape of antheridia, growth rates and optimal growth temperatures for identification purposes. In *Pythium,* the sexual reproductive structures are very important for morphological species identification (Dick, 1990).

Morphological identification of *Pythium* species is problematic for several reasons. Some *Pythium* species fail to produce sexual structures (Van der Plaats-Niterink, 1981). Even if sexual structures are present, high intraspecific variation in the characteristics of these structures can make identification difficult (Matsumoto *et al.*, 2000; Møller & Hockenhull, 2001). For example, some *Pythium* species, such as *P. irregulare* is known to have high intraspecific morphological variation with respect to oogonial ornamentation, oospore size, oogonium size, antheridial cell length and the plerotic state of oospores (Biesbrock & Hendrix, 1967; Van der Plaats-Niterink, 1981; Barr *et al.*, 1997; Matsumoto *et al.,* 2000; Garzón *et al.,* 2007). These factors may lead to errors in identification and sometimes the inability to identify the species.

Problems associated with the morphological identification of *Pythium* species have resulted in molecular techniques becoming very popular for species identification. Some of the earliest studies used restriction fragment length polymorphism analyses of total mitochondrial DNA (Martin, 1989; Martin & Kistler, 1989). Isozyme polymorphisms have

also been used in *Pythium* taxonomy (Barr *et al.*, 1997). More recently, the internal transcribed spacer (ITS) regions have become very popular in the systematics and identification of *Pythium*. The most comprehensive study on the molecular taxonomy of *Pythium* was conducted by Lévesque and De Cock (2004), who constructed an ITS phylogeny of 116 *Pythium* species. This study not only serves as a valuable resource for *Pythium* species identification, but was able to divide the genus into 11 clades (A-K), which mostly correspond to the sporangial morphology of species belonging to specific clades.

In addition to the ITS region, sequence data of some other gene regions, amplified restriction length polymorphisms (AFLPs) and simple sequence repeats (SSRs) have also been investigated for their potential to identify and elucidate the genetic diversity and taxonomy of *Pythium*. A few studies have used sequence data of the the cytochrome *c* oxidase subunit II (*COX2*) (Martin, 2000; Villa *et al.,* 2006) and the β-tubulin gene (Villa *et al.,* 2006; Belbahari *et al.,* 2008; Moralejo *et al.,* 2008). Recently, Robideau *et al.* (2011) and Bala *et al.* (2010) showed that DNA barcoding of the cytochrome *c* oxidase subunit I (*COX1*) and the ITS region can be extremely useful for the identification of oomycetes, including *Pythium*. An extensive sequence database has also been established for *Pythium* (http://www.pythiumdb.org). AFLP data have been used to identify species-diagnostic AFLP fingerprints for nine *Pythium* species (Garzón *et al.,* 2005a) and to identify species boundaries in *P. irregulare* (Garzón *et al.,* 2005b). SSRs were used to investigate the genetic diversity in *P. irregulare* and *P. aphanidermatum* (Lee & Moorman 2008).

Detection and quantification of *Pythium* species. Cultural methods are the traditional manner in which *Pythium* species have been detected and quantified from plant material and soil. This usually involves the direct plating of material onto selective synthetic media (Martin, 1992). Although selective media containing antibiotics and fungicides, such as P_5ARP can be used for the genus *Pythium* (Jeffers & Martin, 1986), several problems can arise when detecting and quantifying *Pythium* from plant material or soil by direct planting. For example, the time used to surface sterilize plant material with disinfectants (e.g. ethanol), which is required for removing saprophytic microbial species growing on the plant surface, should not be too long since this could eliminate the pathogenic *Pythium* species due to penetration of the disinfectant into the infected material (Erwin & Ribeiro, 1996; Van der Plaats-Niterink, 1981). Furthermore, the growth rate of *Pythium* species differs, resulting in

fast growing species being isolated more frequently than slow growing species (Martin, 1992).

Several molecular techniques (DNA-based methods), which are more sensitive than cultural methods and that are also species specific, can be used for the detection and quantification of *Pythium* species. These techniques all have steps that involve the use of the polymerase chain reaction (PCR), which amplifies large copy numbers of certain DNA regions. The DNA regions that are used in detection methods should be sufficiently variable to distinguish the target taxon from related taxa, but sufficiently conserved within the target taxon so that all members of the targeted taxon will be detected (Cooke *et al.,* 2007). In *Pythium,* the DNA region that is mainly used for detection is the ITS region. DNA-based techniques that have been used for the detection of *Pythium* species include (i) DNA-based arrays, (ii) conventional PCR and (iii) quantitative real-time PCR (qPCR).

DNA-based macro-arrays are valuable for the rapid and simultaneous accurate detection of many *Pythium* species from complex environmental samples. The value of DNA-based arrays in *Pythium* detection was first shown by Tambong *et al.* (2006), who developed an array for the detection of more than 100 *Pythium* species. Subsequently, Izzo and Mazzola (2009) developed a DNA array for the detection of a wide range of fungal and oomycetes, including *P. irregulare* and *Pythium* sp. Py26. Zhang *et al.* (2008) also developed an array for several pathogens from solanaceous crops, which included three *Pythium* species (*P. aphanidermatum*, *P. irregulare* and *P. ultimum*).

Quantitative real-time PCR (qPCR) based techniques have several benefits such as increased sensitivity, wider range of quantitative accuracy and less chances for contamination, when compared to conventional PCR and DNA-arrays (Cullen *et al.,* 2007; Vincelli & Tisserat, 2008). Real-time PCR involves a normal PCR, but fluorescently labeled probes, primers or DNA binding dyes, such as Syber Green, are used to monitor fluorescence that is directly related to the quantity of amplicons in the PCR reaction (Okubara *et al.,* 2005). qPCR has been used for the detection of *P. abappressorium* Paulitz & Mazzola,, *P. attrantheridium* Allain-Boulé & Lévesque, *P. heterothallicum*, *P. irregulare* (groups I and V), *P. paroecandrum* Drechsler, *P. rostratifingens* De Cock & Lévesque, *P. sylvaticum* W. A. Campb. & F. F. Hendrix, *P. ultimum* (Schroeder *et al.,* 2006), *P. ultimum* var *ultimum*, *P. vexans* (Spies *et al.* 2011a), *P. oligandrum* Drechsler and *P. dissoticum* Drechsler (Le Floch

et al., 2007). Conventional PCR can also be used for the detection of *Pythium* species, but this is not a quantitative method and only provides information on the presence or absence of a species. *Pythium* species that have been detected using conventional PCR include *P. myriotylum* (Wang *et al.*, 2003) and *P. ultimum* (Cullen *et al.,* 2007).

PYTHIUM SPECIES ASSOCIATED WITH ROOIBOS

Some of the most prominent *Pythium* species that have been found associated with rooibos include *P. acanthicum, P. irregulare, Pythium mamillatum*, *Pythium myriotylum* and *P. pyrilobum* Vaartaja (Bahramisharif *et al.* 2011). These species also play a significant role in crop and yield losses of other hosts such as wheat (*Triticum aestivum* L.) (Higginbotham *et al.,* 2004), soybean (*Glycine max* [L.] Merr), corn (*Zea mays* L.) (Zhang & Yang, 2000) bell pepper (*Capsicum annuum* L.) (Chellemi *et al.,* 2000) and kidney bean (*Phaseolus vulgaris* L.) (Matoba *et al.,* 2008).

Pythium irregulare is a known species complex that fits into *Pythium* clade F *sensu* Lévesque & de Cock (2004). In *P. irregulare*, the ornamented oogonia are irregularly shaped and vary in size. This species usually has monoclinous antheridia and sporangia are rarely formed (Van der Plaats-Niterink, 1981). Several studies have investigated the genetic and morphological variation in *P. irregulare* (Van der Plaats-Niterink, 1981; Barr *et al.,* 1997; Matsumoto *et al.,* 2000; Garzón *et al.,* 2007). Support for the presence of possibly distinct cryptic species within the *P. irregulare* complex is evident from several studies that have used different molecular techniques to characterise isolates, including isozyme polymorphisms, ITS and *COX2* phylogenies, random amplified polymorphic DNA (RAPD) analyses and AFLP analyses (Barr *et al.,* 1997; Matsumoto *et al.,* 2000; Garzón *et al.,* 2007). Following these investigations, Garzón *et al.* (2007) described *P. cryptoirregulare* as a new species within the *P. irregulare* complex. However, Spies *et al.* (2011b) reported that *P. cryptoirregulare, P. cylindrosporum* B. Paul, *P. irregulare* and *P. regulare* Masih & B. Paul may represent only one phylogenetic species. Therefore, the presence of cryptic species in the *P. irregulare* species complex is not yet resolved.

Pythium irregulare has been reported from soil and plant samples worldwide and has a very wide host range (Matsumoto *et al.,* 2000). It was originally isolated from pea (*Pisum*

sativum L.) roots, *Lupinus* and cucumber (*Cucumis sativus* L.) seeds in the Netherlands. This species can be pathogenic to several *Leguminosa, Phaseolus,* ornamentals, and seedlings of other plants (Van der Plaats-Niterink, 1981). Several studies found that *P. irregulare* was the most dominant and prevalent *Pythium* species isolated from different crops (Chamswarng & Cook, 1985; Vincelli & Lorbeer, 1990; Larkin *et al.,* 1995; Pankhurst *et al.,* 1995; Stiles *et al.,* 2007).

Pythium myriotylum was originally described from *Lycopersicon esculentum* L.*,* and has been isolated from several plant species in different regions of the world. This species is known to occur in warmer areas and is not often isolated from temperate climatic zones (Van der Plaats-Niterink, 1981). McCarter and Littrell (1970) showed that *P. myriotylum* was pathogenic towards bean, soybean, rye (*Secale cereal* L.), oats, wheat, peanut (*Arachis hypogaea* L.), sorghum (*Sorghum bicolor* [L.] Moench), tomato (*Solanum lycopersicum* L.) and tobacco (*Nicotiana tabacum* L.). The *P. myriotylum* isolates varied from being highly virulent to only exhibiting low virulence towards each of the crops (McCarter & Littrell, 1970).

Pythium myriotylum has also been found to have intraspecific variation when studied at the molecular level. Perneel *et al.* (2006) reported intraspecific variability in *P. myriotylum* isolates that were isolated from different crops. They used several molecular techniques including esterase banding patterns and AFLPs to show that *P. myriotylum* isolates that were obtained from cocoyam differed from the isolates obtained from other crops. At the molecular level, *P. myriotylum* is difficult to differentiate from *P. zingiberis* M. Takah since these two species have near identical ITS sequences (Matsumoto *et al.*, 1999; Lévesque & De Cock, 2004). These two species can, however, be differentiated morphologically since *P. myriotylum* has aplerotic oospores whereas *P. zingiberis* has plerotic oospores (Lévesque & De Cock, 2004).

Pythium mamillatum was first isolated from *Beta vulgaris* L., and is a very important pathogen because it causes damping-off of several crops (Van der Plaats-Niterink, 1981). Some of the hosts where it causes damping-off include conifer (Vaartaja, 1967 cited by Van der Plaats-Niterink, 1981), kidney bean (Matoba *et al.*, 2008) and *Allyssum* species (Ghaderian *et al.*, 2000). On *Alyssum,* the virulence of *P. mamillatum* is highly influenced by the presence of nickel, with isolates being highly virulent in the absence of nickel, and

virulence being decreased substantially by an increase in nickel concentration (Ghaderian *et al.,* 2000). Hansen *et al.* (1990) found *P. mamillatum* as a predominant species at a conifer nursery in Oregon State.

Pythium pyrilobum is characterized by compound pyriform sporangia, large smooth oogonia and numerous antheridia (Vaartaja, 1965; Van der Plaats-Niterink, 1981). This species was originally reported in Australia from the root collar of a damped-off seedling of *Pinus radiata* (Vaartaja, 1965). Other hosts include papaw (*Carica papaya* L.) (Ward and Shipton, 1984) and rice (*Oryza sativa* L.) (Cother & Gilbert, 1993). On rice, it has been reported to cause a significant reduction in root growth (Cother & Gilbert, 1993) and on papaw it causes root rot (Ward and Shipton, 1984).

Pythium acanthicum differs from most other known species of the genus because of its ornamented oogonia and its contiguous sporangia. This species is pathogenic towards tomato seedlings (Robertson, 1973 cited by Van der Plaats-Niterink, 1981), and also causes blossom-end-rot and fruit rot of water melon (Drechsler, 1939 cited by Van der Plaats-Niterink, 1981). In contrast, Allain-boulé *et al.* (2004) identified *P. acanthicum* as a non-pathogenic species associated with cavity-spot lesions on carrot (*Daucus carota* L.).

PYTHIUM SPECIES ASSOCIATED WITH ROOIBOS ROTATION CROPS (LUPIN AND OATS)

Lupin is an important rotation crop that is used in rooibos nurseries, and it is known to be attacked by several pathogens including *Pythium* spp., causing severe root- or hypocotyl rot and thus reducing the nutrient uptake and grain yields of lupin (Harvey, 2004; Thomas & MacLeod, 2008; MacLeod and Sweetingham, 1997; Sweetingham, 1989). Several *Pythium* species have been recorded as being associated with lupin all over the world, but their pathogenicity was not evaluated. These species include *P. dissoticum* in Queensland (Teakle, 1960 cited by Van der Plaats-Niterink, 1981), *P. intermedium* de Bary in Germany (Schultz, 1939 and 1950 cited by Van der Plaats-Niterink, 1981), *P. rostratum* E.J. Butler in the USA (Middleton, 1943 cited by Van der Plaats-Niterink, 1981) and *P. vexans* in Germany (Schultz, 1939 and 1950 cited by Van der Plaats-Niterink, 1981).

Another important rotation crop of rooibos is oats, where *Pythium* is also an economically important pathogen (Vanterpool & Ledingham, 1930). Several *Pythium* species including *P. myriotylum* (McCarter & Littrell, 1970), *P. torulosum* Coker & P. Patterson (Kilpatrick, 1968 cited by Van der Plaats-Niterink, 1981) and *P. volutum* Vanterpool & Truscott (Vanterpool, 1938 cited by Van der Plaats-Niterink, 1981) are pathogenic towards oats. Welch (1942) reported that *Pythium debaryanum* R. Hesse can cause severe root lesions on oat seedlings in the greenhouse and in the field. In contrast to these pathogenic species, oats are also known to inhibit inoculum production of some *Pythium* species (see under "Rotation crops" section).

MANAGEMENT OF *PYTHIUM*

The management of *Pythium* diseases must consist of an integrated approach, which can include cultural practices (water management, organic amendments and rotation crops), host resistance, fungicide treatments and biological control agents. One of the most reliable and important methods of limiting disease losses to an economically acceptable level is fungicide control strategies. However, fungicides can be detrimental to the environment and human health, and their continued use may result in the development of fungicide resistance (Bruin & Edgington, 1981). Therefore, the use of an integrated management strategy is important, where the reliance on specific fungicides and resistant cultivar lines must be reduced. This will result in the suppression or delay in the development of fungicide resistant strains (Brent & Hollomon, 2007) and strains that can overcome host resistance (Mundt *et al.,* 2002). The use of fungicides is furthermore problematic in organic production. This is especially challenging in rooibos production, where the demand for organic rooibos tea is increasing for international markets and some countries such as Germany and Japan (Personal communication, S. C. Lamprecht, ARC-Plant Protection Research Institute, South Africa).

Chemical control. Fungicides such as the phenylamide fungicides metalaxyl and mefenoxam can provide good control of oomycetes (Erwin & Ribeiro, 1996). Taylor *et al.* (2004) found that mefenoxam could provide moderate control of leak tuber disease caused by *P. ultimum* on potato (*Solanum tuberosum* L.). However, the effect of phenylamide fungicides is not the same for all *Pythium* species based on *in vitro* growth studies, with species such as *P. rostratum* being more sensitive to metalaxyl than *P. torulosum* (Kato *et al.,*

1990). Furthermore, the continuous use of phenylamide fungicides may lead to the development of phenylamide resistant *Pythium* populations (Bruin & Edgington, 1981; Mazzola *et al.,* 2002).

Soil fumigants such as methyl bromide and metham sodium can be used to reduce *Pythium* inoculum in soil (Stephens *et al.*, 1999). As methyl bromide has been phased out in developed countries since 2005, metham sodium and chloropicrin are now widely-used as alternatives to methyl bromide for controlling soilborne fungal and oomycete pathogens (Duniway, 2002; Desaeger *et al.,* 2008). Desaeger *et al.* (2008) found that a combination of 1,3-Dichloropropene (1,3-D), chloropicrin and metham sodium caused a reduction in soil inoculum of *P. irregulare*. Chloripicrin is often applied in combination with 1,3-D, which suppresses pathogenic nematodes. However, this results in a significant loss of flexibility in terms of longer plant-back periods that are required due to the use of 1,3-D products that can have a phytotoxic effect and cause yield losses if the plant-back period is not long enough (Desaeger *et al.,* 2008).

Host resistance. Host resistance is one of the most important control methods that is relatively inexpensive and environmentally friendly. Therefore, breeders have focused on breeding resistant cultivar to reduce losses incited by soilborne pathogens (Bockus & Shroyer, 1998). However, it is more difficult to obtain resistant cultivars against soilborne pathogens such as *Pythium* than for foliar pathogens (Bockus & Shroyer, 1998). Nonetheless, some cultivars in a few crops have been identified that are less susceptible to *Pythium*.

Some of the crops where tolerance to *Pythium* has been identified include soybean and apple rootstocks (Bates *et al.,* 2008; Mazzola *et al* 2009). Bates *et al.* (2008) found that the soybean cultivar Archer is resistant to some *Pythium* species, including *P. aphanidermatum, P. irregulare, P. ultimum* and *P. vexans.* Mazzola *et al.* (2009) evaluated different apple rootstocks and found that the Geneva series rootstocks were less susceptible to *Pythium* species compared to M26, MM106, MM111, Malling and Malling-Merton rootstocks.

Cultural practices.

Water management. Considering the life cycle of *Pythium*, wet conditions play a significant role by inducing sporangial and zoospore production and aiding the movement of zoospores. Therefore, water management is one of the most important considerations in any *Pythium* management strategy (Erwin & Ribeiro, 1996). Over-irrigation and soil compaction should be avoided because they result in water-logged conditions that increase disease incidence (Gubler *et al.,* 2004).

Nutritional amendments. The incidence and severity of *Pythium* infections can also be influenced by the nutritional status of the soil. Calcium could be important in suppressing pathogenic *Pythium* species. In addition to the effect of calcium in suppressing *Pythium*, calcium also has several beneficial effects on the hosts, such as an increase in root production and resistance to infection (Ko & Kao, 1989).

Compost. The use of composts as a soil amendment in horticulture and agriculture is attractive since (i) it may lead to a reduction in the use of non-renewal inorganic fertilizers, (ii) it may suppress disease due to its effects on soil microbial communities and (iii) can contribute to the recycling of waste (Termorshuizen *et al.,* 2006; Biswas and Narayanasamy, 2006; De Ceuster & Hoitink 1999). Compost is specifically attractive as a fertilizer since it contains nitrogen, phosphorus, calcium and organic matter (Iqbal *et al.,* 2010; Lewis *et al.,* 1992). It is also a soil conditioner that enhances aeration and water status, thus improving soil quality (Amlinger *et al.,* 2007).

The feedstocks from which compost are made off, and the degree to which it is composted, may have an effect on the suppressive nature of composts towards *Pythium* damping-off (Termorshuizen *et al.,* 2006; Ben-Yephet & Nelson, 1999). Composts are made from a broad range of raw feedstocks, including green and yard waste, straw, bark, biowaste and municipal sewage that can be composted to various degrees. Although, Hoitink & Boehm (1999) found that composted materials are more suppressive than uncomposted feedstocks to *Pythium* root rot, Aryantha *et al.* (2000) found composted and uncomposted animal manures to be equally suppressive towards *Phytophthora cinnamomi* Rands on lupin. Erhart *et al.* (1999) reported that compost made from bark was significantly suppressive towards *Pythium*

diseases, but compost prepared from biowaste had no significant effect on the suppression of disease.

The level of *Pythium* disease suppression achieved with compost could be caused by different biological mechanisms. Several studies have indicated that suppression of Pythium damping-off is associated with the level of microbial activity and -biomass in compost-amended container media (Chen *et al.,* 1988a; Chen *et al.,* 1988b; Hadar & Mandelbaum, 1986; Mandelbaum *et al.,* 1988). For example, Chen *et al.* (1988a) and Craft and Nelson (1996) identified a negative correlation between Pythium damping-off severity and compost microbial biomass (as measured by the hydrolysis of fluorescein diacetate). Some composts can, however, increase the severity of oomycete induced diseases. Hoitink *et al.* (1997) reported that composts high in saline enhance *Pythium* and *Phytophthora* diseases. Therefore, the effect of organic amendments such as compost should be carefully evaluated before being implemented in an integrated management strategy.

Green manures, Brassica green manures and Brassica seed meals. Green manures provide some benefits to the soil such as increasing nutrients and organic matter, improving soil structure, and reducing soil erosion (Abdallahi and N'Dayegamiye, 2000; Al-Khatib *et al.,* 1997; Blackshaw *et al.,* 2001). Although green manures can suppress pathogens, their application may also result in coincidental negative effects such as an increase in disease incidence and severity, especially if the plant back period is too short after green manure incorporation (Bonanomi *et al.,* 2009). For example, the application of green manures to nitrogen fixing species, e.g. *Vicia sativa* L., which releases ammonia during residue decomposition, could enhance the incidence of *Pythium* spp. (Rothrock & Kirkpatrick, 1995).

Brassica green manures have been recognized as a potential control strategy for soil-borne pathogens (Wiggins & Kinkel, 2005; Walker & Morey, 1999; Mazzola, 2003; Mazzola *et al.,* 2006; Njoroge *et al.,* 2008). In addition to Brassica green manures, Brassica seed meal, which is a waste product of biodiesel production, can also be used for suppressing *Pythium* (Mazzola *et al.,* 2006). Brassica green manures are incorporated into the soil at flowering and release toxic volatile compounds, such as isothiocyanates generated from the glucosinolates in these crops. The released volatiles result in a fumigation effect, known as biofumigation, which contributes to the control of nematodes and soilborne pathogens, such as *Fusarium, Rhizoctonia* and *Cylindrocarpon* species (Stephens *et al.*, 1999; Walker &

Morey, 1999; Mazzola *et al.*, 2001; Bello *et al.*, 2004; Oka, 2009). The fumigation effect is not always the mechanism involved in disease suppression, since the amendment of soil with Brassica crop residues or seed meals can result in a shift in microbial population structure towards a population that is beneficial for plant growth with deleterious communities being less prominent (Mazzola *et al.*, 2006). Several reports have been published where *Pythium* populations increase with the use of certain biofumigants or green manures (Stephens *et al.*, 1999; Walker & Morey, 1999; Mazzola *et al.,* 2001; Manici *et al.,* 2004).

Different Brassica species can have a differential effect on the suppression of *Pythium*. Although the application of *B. napus* L. seed meal induced an increase in *Pythium* populations (Cohen *et al.,* 2005; Mazzola, 2003; Mazzola *et al.,* 2007), *B. juncea* L. seed meal did not stimulate *Pythium* apple orchard soil populations (Mazzola *et al.,* 2007). Consequently, amendment of apple replant soil with *B. napus* significantly reduced the growth of apple seedlings, whereas amendment of replant soil with *B. juncea* seed meal resulted in a significant increase in apple seedling growth. Co-application of *B. napus* with *B. juncea* seed meal amendments also did not stimulate pathogenic *Pythium* populations, but improved apple seedling growth. This strategy also resulted in the combined suppression of *Pythium* and another apple replant pathogen, *Rhizoctonia solani* Kühn (Mazzola *et al.,* 2007). Walker & Morey (1999) also found large increases in soil *Pythium ultimum* population sizes when using *B.* juncea *and B. napus* ssp. *oleifera biennis.*

Rotation crops. Crop rotation is an important and widely used cultural practice that can suppress several different soilborne diseases. The use of non-host rotation crops may prevent the development of large populations of soilborne pathogens, since they reduce the selection for specific soilborne pathogens. Another benefit of rotations that include grasses and legume sods, is that they can increase soil fertility, due to their enhancement of soil nutrient balances and the addition of organic matter to soil (Fry, 1982; Havlin *et al.,* 1990). There are, however, some constraints that prevent the wide-scale use of crop rotation in plant disease management (Fry, 1982). For example, some soilborne pathogens such as *P. irregulare* has a very broad host range (Van der Plaats-Niterink, 1981), which restricts the number of crops that can be used as rotation crops (Fry, 1982).

Depending on the rotation crop used, rotation may or may not provide disease control and can even increase the incidence of certain diseases due to the broad host range of some

Pythium species. Davis and Nunez (1999) found a significant increase in carrot root dieback caused by *P. aphanidermatum, P. irregulare* and *P. ultimum*, when carrots followed alfalfa and barely. Ingram and Cook (1990) were also unable to show that crop rotations that include wheat, spring barley, lentils and peas can reduce the pre-emergence and post-emergence damping-off of these crops caused by *P. irregulare* and *P. ultimum* var. *sporangiiferum*. In contrast, Davison & McKay (2003) reported a significant reduction in the incidence and severity of cavity spot of carrots caused by *Pythium* spp., including *P. sulcatum*, when carrots were followed by one or more broccoli crops. Mazzola and Gu (2000) showed that the use of a wheat cover crop during apple orchard renovation could reduce root infections caused by *Pythium* species, and thus lead to improved apple growth in replant soils. It was important to note that wheat cultivars differed in their suppressive effect (Mazzola & Gu, 2000). Therefore, the selection of the correct cultivar of a specific rotation crop may also be important for achieving disease suppression.

Oats can be used as a rotation crop to reduce the incidence and severity of root rot diseases caused by soilborne pathogens including some *Pythium* species (Williams-Woodward *et al.,* 1997). The bio-control effect of oats was demonstrated clearly in a study by Deacon & Mitchell (1985). They found that oat roots are effective in suppressing oomycetes, since they attract and cause lysis of zoospores of several *Pythium* species (*Pythium aphanidermatum, Pythium arrhenomanes* Drechsler, *Pythium graminicola* Subramaniam, *Pythium intermedium, Pythium 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.

Physical methods. Physical agents such as heat, soil solarization and radiation can only be used as pre-plant soil treatments since they can be harmful to the crop of interest. Although physical methods can be effective in suppressing soilborne pathogens, their use may be complicated by several factors including costs, safety, technological problems and difficulty in reaching the inoculum at all soil sites and depths (Katan, 2000). In general, soil solarization kills soilborne pathogens through the generation of high soil temperatures that cannot be tolerated by the pathogens. Disease suppression may also be caused by an increased frequency of antagonistic bacteria (*Bacillus* spp. and *Pseudomonas fluorescens*) in the rhizosphere of plants grown in solarized soil (Stapleton *et al.,* 1987; Stapleton and DeVay, 1984; Gamliel and Katan, 1993).

There are not many studies that have investigated the effect of solarization on *Pythium* diseases. A study by Gamliel and Stapleton (1993) reported that solarization had a significant effect on the reduction of *P. ultimum* in fall and spring lettuce crops. The solarization treatments were also effective at increasing the yield of lettuce.

Biological control. Biocontrol agents may play a significant role in the control of several plant pathogens including oomycetes. Numerous organisms have been identified as having the potential for suppressing *Pythium,* including *Enterobacter cloacae* (Jordan) Hormaeche and Edwards, *Gliocladium virens* Miller *et al., Pseudomonas fluorescens* (Trevisan) Migula*, Pythium acanthicum* Drechsler, *P. oligandrum* Drechsler*, P. periplocum* Drechsler*, Streptomyces griseoviridis* Anderson *et al., Trichoderma koningii* Oud, and *T. harzianum* Rifai on various crops (Hadar *et al.,* 1984; Whipps & Lumsden, 1991; Ribeiro & Butler, 1995; Ali-Shtayeh & Saleh, 1999; Quagliotto *et al.,* 2009). *Trichoderma* harzianum and *T. koningii* have been shown to reduce various crops affected by *Pythium* damping-off including beans, cucumber and peas (Hadar *et al.,* 1984, Sivan *et al.,* 1984, Naseby *et al.,* 2000). However, the efficacy of biological control often varies between different sites due to the complex nature of the soil environment. Therefore, the use of such agents at a commercial scale is limited (Handelsman & Stabb, 1996).

CONCLUSION

Many biotic and abiotic components contribute to a reduction in rooibos production. One of the most important biotic factors is damping-off caused by oomycetes. Currently, these pathogens can be controlled using fungicides, but their use is not allowed in organic production. The production of organic rooibos is important, since rooibos tea is becoming very popular in European countries, due to its high level of antioxidants, its low tannin levels and its lack of caffeine. Therefore, in order to produce rooibos organically, alternative management practices to fungicide treatments should be investigated to protect rooibos seedlings from oomycete damping-off. Promising management strategies could include the amendment of soil with compost, a rotation crop such as oats, and the use of non-pathogenic *Pythium* species.

The development of alternative management strategies will require knowledge of the specific oomycete species that are involved in rooibos damping-off. It is therefore important to identify the specific oomycete species involved in damping-off. This knowledge will allow the development of molecular techniques such as DNA macro-arrays and quantitative PCR (qPCR) for the rapid assessment of the species involved, and the quantification of inoculum in nursery soils. These techniques can also be used to investigate the mechanisms involved in disease suppression by specific management practices. For example, if a specific management strategy causes a shift from pathogenic oomycete species to beneficial oomycete species.

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2. PUTATIVE NEW *PYTHIUM* SPECIES FROM CLADE G ASSOCIATED WITH THE SOUTH AFRICAN INDIGENOUS PLANT *ASPALATHUS LINEARIS* (ROOIBOS)

ABSTRACT

The genus *Pythium* consists of more than 120 species, and has been subdivided into eleven phylogenetic clades (A to K) based on internal transcribed spacer (ITS) region sequence data. A total of 12 *Pythium* isolates were obtained from the indigenous plant *Aspalathus linearis* (rooibos) in South Africa that fit into clade G. *Pythium* clade G only contains six known species, with the morphological descriptions of several species being imprecise and confusing. Therefore, the aims of this study were to characterize the 12 clade G isolates that were obtained from rooibos, re-investigate the reference isolates of five of the known clade G species and develop oligonucleotides for differentiating two rooibos groups that may represent new species, using DNA macroarray analyses. Phylogenetic analyses of the internal transcribed spacer (ITS) regions and a combined phylogeny of four gene regions (ITS, β-tubulin and, *COX1* and *COX2* [cytochrome c oxidase subunits I and II]) identified five sub-clades (1-5) within *Pythium* clade G. The rooibos isolates formed two groups, *Pythium* Rooibos group I (RB I) and II (RB II) that clustered in sub-clade 1a (76%-89% bootstrap, 1.00 probability) and 1b (70%-64% bootstrap, 1.00 probability) respectively. The *Pythium* RB I isolates formed a distinct clade from *P. iwayamai*, and may represent a new species. The *Pythium* RB II isolates, all from different locations, had *P. canariense* and *P. violae* as their closest relatives and may also represent new species. Genetic variation within sub-clade 1b, which contained the *Pythium* RB II isolates, suggested the presence of several new species. Morphological analyses of the rooibos isolates were inconclusive, since the isolates all contained similar morphological characteristics that did not correspond to the descriptions of *P. iwayamai*, *P. violae* or *P. canariense.* The clade G reference isolates were not useful for morphological comparisons since the isolates were all sterile. *Pythium* RB I and RB II are not described here as new species, since representatives of their closest relatives (*P. iwayamai*, *P. violae* and *P. canariense*) are limited, which precludes investigations into the intraspecific variation within and across sub-clades in *Pythium* clade G. Furthermore, the morphological description of most current species is confusing and imprecise, precluding morphological comparisons. Future clade G studies should include

more isolates representing the diversity within each sub-clade, good morphological descriptions and a multigene phylogeny. The *Pythium* RB I and II isolates were all non-pathogenic toward rooibos, lupin and oats seedlings. One oligonuleotide was developed for each of *Pythium* RB I and RB II, which was able to differentiate the isolates from each other, and from clade G reference isolates using DNA macroarray analyses.

INTRODUCTION

The genus *Pythium* contains more than 120 species that have a range of pathogenic to beneficial interactions with plants (Alexopoulos *et al.*, 1996; Dick, 2001). Due to the presence of pathogens and non-pathogens within the same genus, the correct species identification is important. *Pythium* species identification can be conducted using morphological and molecular methods. Morphological identification is very difficult due to the (i) high intraspecific variation in morphological characteristics (Martin, 2000), (ii) lack of consensus about the importance of different structures in morphological identification (Matsumoto *et al.*, 1999), (iii) morphological characteristics overlapping among species and (iv) many *Pythium* species not forming sexual structures, which are most important for species identification (Van der Plaats-Niterink, 1981). Therefore, molecular identifications, specifically internal transcribed spacer region (ITS) sequence data, are important for accurate species identifications. The most comprehensive study on *Pythium* species identification was conducted by Lévesque & De Cock (2004), who constructed an ITS phylogeny of 116 *Pythium* species, which subdivided the genus into 11 clades (A-K).

Some of the *Pythium* clades identified by Lévesque & De Cock (2004) have been studied in more detail than others, for example, several publications are available on clade F species (Matsumoto *et al.*, 2000; Garzón *et al.*, 2005, 2007; Spies *et al.*, 2011a), whereas less is known about clade G species. Clade G is a small clade that only contains six known species including *Pythium iwayamai* S. Ito, *P. nagaii* S. Ito & Tokunaga, *P. okanoganense* Lipps, *P. paddicum* Hirane, *P. violae* Chesters & Hickman (Lévesque & De Cock, 2004) and *P. canariense* (Paul, 2002). Representative GenBank sequences and isolates of these species and other related putative new taxa are also very limited. The phylogenetic clustering of some clade G sequences can also be confusing, for example, some *P. iwayamai* sequences that sometime cluster within the *P. okanoganense*/*P. paddicum* sub-clade (Lévesque & De Cock,

2004, McLeod *et al*., 2009). Taxonomical analyses of clade G is further complicated by imprecise morphological descriptions of *P. canariense* (Paul, 2002), *P. iwayamai* (Iwayama, 1933; Ito & Tokunaga, 1935; Hirane, 1960) and *P. violae* (Chesters & Hickman, 1944). It is clear that clade G is in need of a major taxonomical revision employing both morphological and phylogenetic data.

Phylogenetic species recognition involves the use of phylogenetic analyses of gene sequences for recognizing species. Although this approach is more reliable and accurate than traditional morphological and/or biological species recognition, the use of inadequate sequence data can result in incorrect species identification or delineation. Although many studies in *Pythium* taxonomy only relied on the ITS region, this region cannot always differentiate between the known *Pythium* species and cryptic species (Lévesque & De Cock, 2004; McLeod *et al.*, 2009). To address such problems, Taylor *et al.* (2000) proposed the use of multiple genes in an approach called genealogical concordance phylogenetic species recognition (GCPSR) whereby phylogenetic species are recognized based on the concordance of multi-gene phylogenies. Aside from ITS, other gene regions that have been used to a limited extend for species recognition in *Pythium* include the cytochrome *c* oxidase subunit I and II (*COX1*, *COX2*), and β-tubulin (Martin, 2000; Garzón *et al.*, 2005, 2007; Villa *et al.,* 2006; Belbahri *et al.,* 2008; Lévesque *et al.,* 2008; Moralejo *et al.*, 2008; Spies *et al.*, 2011a, 2011b; Bala *et al.*, 2010 Robideau *et al.*, 2011). Of these, the ITS and mitochondrial *COX2* regions have proven useful in several taxonomic studies (Matsumoto *et al.*, 1999; Martin, 2000; Lévesque & De Cock, 2004). More recently, the *COXI* and ITS regions have been used successfully as DNA bar-coding regions for the identification of *Pythium* species (Robideau *et al.*, 2011; Bala *et al.*, 2011), which also generated more sequence data for constructing multiple gene phylogenies. In contrast, the β-tubulin region has only been used in a few phylogenetic studies of the genus *Pythium* (Villa *et al.*, 2006; Spies *et al.*, 2011a, 2011b).

Pythium damping-off of rooibos (*Aspalathus linearis* (N.L. Burm.) R. Dahlgr.), an indigenous crop in South Africa that is mainly used to produce rooibos tea, is causing severe losses in nurseries. *Pythium* species are also involved in damping-off and root rot of lupin (*Lupinus angustifolius* L.) and oats (*Avena sativa* L.) (Sweetingham, 1989; McCarter & Littrell, 1970), two rotation crops used in rooibos nurseries. Preliminary work has shown that rooibos harbors various *Pythium* species, including isolates that fit into clade G (Bahramisharif *et al.*, 2011). The clade G isolates have not been characterised with regards to

species identity and pathogenicity toward rooibos and the two nursery rotation crops, which is important for the development of sound management strategies. In rooibos nurseries, the identification of *Pythium* species from complex soil and plant samples through isolation studies has proven to be time consuming, and can currently not be done on a routine basis for making management decisions. DNA-based technologies could offer a solution to this problem. For example, a *Pythium* DNA-macroarray based on the ITS region has been developed by Tambong *et al.* (2006). The DNA-macroarray can rapidly identify most species in one assay since it contains 172 oligonucleotides that recognize more than 100 of the known species (Tambong *et al.*, 2006). The DNA macroarray should preferably not only identify known species, but also all of the undescribed *Pythium* taxa identified from rooibos.

The aims of the study were to characterize *Pythium* clade G isolates that were previously obtained from rooibos nurseries and rooibos plants growing in a natural ecosystem (Bahramisharif *et al.*, 2011). The isolates were characterised morphologically, phylogenetically (ITS, β-tubulin, *COX*1, and *COX2* regions) and for their pathogenicity toward rooibos, lupin and oats. Reference isolates that represented five of the known clade G species (*P. canariense, P. iwayamai, P. nagaii, P. paddicum* and *P. violae*) were also characterised morphologically and phylogenetically. Lastly, for two *Pythium* rooibos groups (*Pythium* RB I and RB II) that may represent new clade G species, oligonucleotides were designed to differentiate these two groups from other known clade G species using DNA macroarray analyses.

MATERIALS AND METHODS

***Pythium* isolates.** Twelve *Pythium* isolates were obtained in a previous study from rooibos nurseries and mature rooibos plants in a natural ecosystem (Bahramisharif *et al.*, 2011). The isolates are available from the Stellenbosch University culture collection, and their origin is shown in Table 1. Most of the Clade G reference isolates that represented five of the known clade G species were sourced from the Centraalbureau voor Schimmelcultures, the Netherlands (CBS codes), or from the Plant Protection Research Institute (Agriculture Research Council, Plant Protection Research Institute, Pretoria, South Africa) culture collection (PPRI codes). These isolates included *P. iwayamai* (CBS 156.64) and *P. violae* (CBS 159.64), which were the strains examined in the monograph of the genus *Pythium* by

van der Plaats-Niterink (1981), *P. canariense* ex-holotype strain (CBS 112353), *P. paddicum* (CBS 698.83) from the study of Lévesque & De Cock (2004). Other isolates that were also included in the study were *P. iwayamai* (CBS 697.83) from the study of Lévesque & De Cock (2004), *P.* sp. *WJB-1* (PPRI8300) from the study of McLeod *et al.* (2009), *P. nagaii* (P16712) was obtained from M.D. Coffey (University of California Riverside, Department of Plant Pathology and Microbiology) and *Pythium* aff. *canariense* (OW1707) that has similarity to *P. canariense* and was obtained from C.F.J. Spies (Stellenbosch University, Department of Plant Pathology). This isolate is no longer available in culture and has died in storage. All isolates were stored as CMA culture plugs in sterile de-ionised water containing grass blades, as V8-agar (Galindo & Gallegly 1960) plugs in sterile de-ionised water, as well as potato-carrot agar (Dhingra & Sinclair 1985) slant cultures at 15 °C.

DNA extraction, PCR amplification and sequencing. All *Pythium* isolates were grown on V8 agar for 5-7 days. Aerial mycelium was harvested and genomic DNA was extracted using a slightly modified protocol of Lee and Taylor (1990) (Tewoldemedhin *et al.*, 2011). Sequencing analyses were conducted for all the isolates for four gene regions (ITS, *COX1*, *COX2*, and β-tubulin) (Spies *et al.*, 2011b) by the Central Analytical Sequencing Facility at Stellenbosch University using the BigDye terminators system (version 3.1, Applied Biosystems) and an ABI 3130XL Genetic Analyzer (Applied Biosystems).

The ITS sequence data obtained for isolate STE-U 7558 was of poor quality, and required cloning. The PCR products of the isolate were cloned using InsTAclone™ PCR Cloning Kit (Fermentas Inc., Glen Burnie, MD) according to the manufacturer's instruction. Two clones were chosen for sequencing.

Phylogenetic analyses. The ITS sequences of the rooibos isolates were used in GenBank BLAST analyses for identifying sequences that were closely related to these sequences. The sequences that were identified included *P.* aff. *canariense* (OW1707, JF499669), *P. nagaii* (CBS 779.96, AY598705), *P. okanoganense* (CBS 701.83, AY598718), *P. okanoganense* (CBS 315.81, AY598649), *P.* sp. *6 eu* (EU038812.1), *P.* sp. *OPU1336* (AB299394.1), *P.* sp. *OPU1435* (AB299395.1), *P.* sp. *PB-2007* (AW489757.1), *P. paddicum* (AB217667.1) and *P. Ro21* (AY129553.1). Sequences of the known clade G species were also included in the phylogeny.

The sequences of each gene region were aligned using MAFFT sequence alignment program version 6 (Katoh & Toh 2008). The alignments were manually adjusted in Geneious Pro v5.5 (Drummond *et al*., 2010) for improved alignment. *Pythium ultimum* Trow var. *ultimum* (PPRI8615) and *Pythium helicandrum* Drechsler (PPRI8508) were used as outgroups in all the phylogenies. Maximum parsimony analysis (MP) and Bayesian analysis were conducted.

The MP analyses were generated using PAUP* (Phylogenetic Analysis Using Parsimony) v. 4.0b10. The analysis was performed using the heuristic search option with 100 random addition replicates. Tree bisection and reconstruction (TBR) was used as the branch swapping algorithm with the option of saving no more than 10 trees with a score greater than or equal to 5. Alignment gaps representing putative deletion sites were treated as missing data. All characters were weighted equally and unordered. Equally parsimonious trees were saved. Bootstrap support values were calculated from 100 heuristic search replicates. Other measures calculated for parsimony included tree length (TL), consistency index (CI), retention index (RI) and the rescaled consistency index (RC) values. Partition homogeneity tests were conducted and datasets were combined, when *P*-values were greater than 0.05.

Bayesian analyses were performed using MrBayes v. 3.0b4 (Ronquist & Huelsenbeck 2003). The optimal model of sequence evolution was selected by using the program MrModeltest (Posada & Crandall, 1998) as previously described (McLeod *et al.,* 2009). The selected model for each region was as follows: HKY+G for the ITS, GTR+G for the β-tubulin and GTR+I+G for the *COX1* and *COX2*. The first 125000, 19100 generations (burn-in) were discarded for the ITS and the combined ITS, β-tubulin, *COX1* and *COX2* analyses, respectively. The remaining samples were used to calculate the 50% majority-rule tree and the posterior probability for the individual branches.

Morphological characterisation. The 12 rooibos isolates, and isolates of the known species that are either ex-holotype or isolates used by Van der Plaats-Niterink (1981) including *Pythium canariense* (CBS 112353), *P. iwayamai* (CBS 156.64), *P. nagaii* (P16712), *P. paddicum* (CBS 698.83) and *P. violae* (CBS 159.64) were characterised morphologically. Additionally *P. iwayamai* CBS 697.83 was also included. The isolates were sub-cultured on CMA supplemented with 30 mg/l β-sitosterol and incubated for 3-5 d at 25 °C in the dark. Five inoculum plugs (5 mm^2 diam) of each isolate were plated in sterile 90-

mm-petri dishes containing sterile soil-water extract (20 g sandy soil suspension in 1 l distilled water, filtered and autoclaved at 121 °C at 15 kPa for 20 min). Sterile grass blades (2 cm length) (*Pennisetum clandestinum* Hochst. ex Chiov) were floated on the sterile soil extract and incubated at 25 °C under cool white fluorescent light using 12 h light and 12 h darkness until sporulation was observed. Plates were examined and measurements were made for sporangia, hyphal bodies, chlamydospores and oogonia under a compound light microscope at 60× and 400× and 1000× magnifications, using differential interference contrast illumination. Digital images of oogonia were taken with a Zeiss digital camera (Axiocam MRc) and Axiovison release 4.8.2 software (Carl Zeiss, AG, Oberkochen, Germany). Original species descriptions and the dichotomous keys of Dick (1990) were consulted to compare morphological characteristics observed on plates and in soil-extract cultures. The monograph of the genus *Pythium* (Van der Plaats Niterink, 1981) was also used for species descriptions. Sexual and asexual morphological structures in the culture including biometric data were used for species identification, as suggested by Dick (1990) and Shahzad *et al.* (1992).

Design of specific oligonucleotides for DNA macroarray analyses. The phylogentic analyses showed that the rooibos isolates grouped into two distinct groups, *Pythium* Rooibos group I (RB I) and II (RB II) (see results section). The ITS sequences of the two *Pythium* RB groups were aligned using Geneious Pro v5.5. Sequences of *P. canariense* (CBS 112353), *P. iwayamai* (CBS 156.64), *P. iwayamai* (CBS 697.83), *P. nagaii* (P16712), *P. okanoganense* (CBS 701.83), *P. okanoganense* (CBS 315.81) *P. paddicum* (CBS 698.83), *P.* sp. *OPU1336* (AB299394.1), *P.* sp. *OPU1435* (AB299395.1), *P. Ro21* (AY129553.1), *P.* sp. *PB-2007* (AW489757.1), *P.* sp. *6 eu* (EU038812.1), *P. violae* (CBS 159.64) that are closely related to these groups were also included in the alignment. Unique polymorphisms were identified visually, and were used to design four oligonucleotides (spiwa1, spiwa2, spcan1 and spcan2), specific for each of the two Rooibos groups (Table 2). The lengths of the newly designed oligomers were between 17 and 27 nucleotides and the estimated melting temperature (Tm) ranged between 50 °C to 60 °C. The formula used to calculate Tm was: $Tm = 64.9 + 41 \times (y + z - 16.4)/(w + x + y + z)$, where w, x, y, and z are the number of the bases A, T, G, and C in the sequence, respectively (Howley *et al.,* 1979).

DNA macroarray analyses. The macroarray membranes were produced by first diluting oligonucleotides (Sigma-Aldrich, Steinheim, Germany), which was synthesised with

a C6 5' amino group, to 40µM in sodium hydrogen carbonate buffer (pH 8.0) (Tambong *et al.*, 2006). The oligonucleotides (2 µL) were spotted in duplicate onto Immunodyne ABC membranes (Pall Europe Limited, Portsmouth, England) using a multi-pipette (Biohit, Helsinki, Finland). The replicate spots of each oligonucleotide were placed below each other on a diagonal (45° offset). In addition to the four *Pythium* RB I and RB II oligonucleotides, 54 olignonucleotides from the study of Tambong *et al.* (2006) were also spotted onto the membrane and included the (i) clade G oligonucleotides iwa51 (*iwayamai* group), iwa52 (*P. iwayamai*), and vioIwa (*P. violae, P. iwayamai*), (ii) 12 oligos that detect other *Pythium* species that have been identified from rooibos (Bahramisharif *et al.*, 2011; Chapter 3) and (iii) nine oligos that detect pathogenic *Pythium* species that are frequently found in South Africa, e.g. *P. aphanidermatum* (Edson) Fitzp., *P. ultimum* and *P. vexans* de Bary. The spotted membranes were air dried for 10 min and transferred to blocking solution (20× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.5% skim milk powder [Difco, LePont de Claix, France] and 0.05% Tween 20) with agitation for a minimum of 15 min. Membranes were rinsed briefly in 2× SSC, air dried and kept at room temperature in a plastic bag until use.

The macroarray membranes were hybridized with ITS amplicons obtained from two *Pythium* RB I isolates (STE-U 7555 and STE-U 7556), three *Pythium* RB II isolates (STE-U 7548, STE-U 7549 and STE-U 7550), *P. canariense* CBS112353, *P. iwayamai* CBS156.64 and *P. viola* CBS 159.64. The ITS amplicons were amplified with primers ITS6 and ITS4 as described above, and were labeled and hybridized using the Gene Images AlkPhos Direct Labeling and Detection System with CDP-*Star* (Amersham Biosciences, NJ) following the manufacturer's protocol. Briefly, the array was pre-hybridized at three different temperatures (50, 55, and 60 °C) for 90 min, followed by hybridization with 250 ng of labeled ITS amplicon at three different temperatures (50, 55, and 60 °C) for 12 h. After hybridization, membranes were washed four times in primary and secondary wash buffers, and detection reagent was applied to the membrane for 4 min, followed by 24 h of film exposure. Chemiluminescence was detected using Kodak Biomax Light film (Sigma-Aldrich, Steinheim, Germany).

Pathogenicity towards rooibos, lupin and oats. The pathogenicity of three isolates of *Pythium* RB I and three isolates of *Pythium* RB II was determined towards rooibos, lupin,

and oats in a glasshouse trial. Inoculum was produced by using a sand-bran inoculation method (Lamprecht, 1986). Briefly, 30 ml of water was added to a mixture of washed sand and wheat bran (200 g sand, 10 g wheat bran) in 1 l glass bottles. The mixtures were autoclaved three times, and subsequently inoculated with ten 5 mm diameter plugs of 6-day-old PDA cultures of different isolates (Lamprecht & Tewoldemedhin, 2011). The uninoculated control consisted of sand and wheat bran that were inoculated with un-colonized agar plugs. The inoculated bottles were incubated at 25 °C and were shaken three times a week to ensure the colonization of the media over the 10 day incubation period.

Plastic pots (13 cm diameter) were filled with 800 g of planting medium. The planting medium consisted of equal quantities of soil, perlite and sand, which was pasteurized for 30 min at 80 °C for three consecutive days. Each pot with medium was then inoculated with an inoculum concentration of 0.05% of the respective isolates. Rooibos (50 seeds per pot), oats (50 seeds per pot) and lupin (20 seeds per pot) seeds were planted in the pots. For each isolate, three replicate pots were included in the trial. The pots were arranged in a randomized block design. The experiment was carried out in the glasshouse at approximately 18 °C night and 27 °C day temperatures. Negative controls consisting of un-colonized sand-bran were also included. The experiment was conducted twice.

The pathogenicity of isolates was determined by measuring seedling length, and the percentage survival. The percentage survival of rooibos, oats, and lupin seedlings was determined after 2 weeks. The length was calculated for oats and lupin after 2 weeks, and for rooibos after 4 weeks. Re-isolations for *Pythium* were made from the seedlings to fulfill Koch's postulates as previously described (Tewoldemedhin *et al.*, 2011).

Statistical analyses. Levene's variance ratio test was performed for homogeneity of trials (Levene, 1960). Data were subjected to analysis of variance using SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC, USA) and the Shapiro-Wilk test was used to test for normality (Shapiro & Wilk, 1965). Fisher's least significant difference (LSD) was calculated for each of these parameters (Ott, 1998).

RESULTS

Phylogenetic analyses.

ITS phylogeny. The ITS phylogeny revealed five sub-clades (numbered 1-5) in *Pythium* clade G (Fig. 1). The first sub-clade (93% bootstrap, 1.00 probability) could be further subdivided into sub-clades 1a (74% bootstrap, 1.00 probability) and 1b (70% bootstrap, 1.00 probability). Sub-clade 1a contained sequences from nine rooibos isolates (including both polymorphic sequences from isolate STE-U 7558 [JQ412774 and JQ412775]) in a single well-supported clade (100% bootstrap, 1.00 probability; hereafter referred to as Rooibos group I), which is distinct from the sequence of *P. iwayamai* CBS 156.64 (AY598648). The polymorphic sequences for isolate STE-U 7558 only differed by 2 bp and 2 bp indels. Sub-clade 1b contained sequences of the other three rooibos isolates (hereafter referred to as Rooibos group II), a GenBank sequence of *P.* aff. *canariense* (OW7107, JF499669), *P. violae* CBS 159.64 (AY598706) and the ex-holotype strain of *P. canariense* (CBS 112353, HQ665069). Genetic variation within sub-clade 1b was apparent from varying branch lengths and further subdivisions with good support. Sub-clade 2 (85% bootstrap, probability < 0.60) contained two GenBank sequences of putative new species, *P.* sp. 6 eu (EU038812) and the South African isolate *P.* sp. WJB-1 (FJ415946) (McLeod *et al.*, 2009). Sub-clade 3 (100%, 1.00 probability) contained four GenBank sequences of yet another putative new species referred to in GenBank as *P.* sp. OPU1336, *P.* sp. OPU1435, *P.* sp. Ro21 and *P.* sp. PB-2007 (AB299394, AB299395, EU038812, AY129553, and AM489757). Sub-clade 4 (100% bootstrap, 1.00 probability) contained the type strain sequence of *P. okanoganense* (CBS 315.81, gi51235503) as well as sequences of two isolates each of *P. paddicum* (CBS 698.83 [AY598707] and AB217667) and *P. iwayamai* (CBS 697.83, AB299388). Sub-clade 5 contained two sequences of *P. nagaii* (CBS 779.96 [AY598705] and P16712).

Four gene combined phylogeny. Partition homogeneity tests showed that the data-sets of the ITS, *COX*1, *COX*2 and ß-tubulin loci were congruent ($P > 0.05$ for all possible combinations). Sequence data for all four loci were consequently combined into a single dataset for phylogenetic analyses. The combined phylogeny (Fig. 2) supported sub-clades 1, 2, 4 and 5 identified in the ITS phylogeny (100% bootstrap and 1.00 probability for each clade with more than one representative). ITS sub-clade 3 was not included in the combined

analyses due to lack of sequence data for the *COX1*, *COX2* and ß-tubulin regions. The subdivision of sub-clade 1 into sub-clades 1a and 1b was well-supported in the Bayesian analysis (1.00 probability for both sub-clades 1a and 1b), but while sub-clade 1a had good bootstrap support in the parsimony analyses (89%), sub-clade 1b did not (64% bootstrap). As in the ITS phylogeny, *Pythium* RB I formed a well supported clade in sub-clade 1a (100% bootstrap, 1.00 probability) that was distinct from *P. iwayamai* CBS 156.64. However, where *P.* aff. *canariense* OW1707 clustered within the *Pythium* RB II clade in the ITS phylogeny, it was distinct from this group in the combined phylogeny. As in the ITS phylogeny, differing branch lengths and sub-clade structuring revealed genetic diversity within sub-clade 1b.

Morphological characterisation. The morphological characteristics of all *Pythium* RB I isolates were similar (Fig. 3). The isolates did not form sporangia, but appressoria were observed. Mating system homothallic. Oogonia mostly terminal, but sometimes intercalary (av. 22 µm diam), smooth, some mono or multi-papillate, wall can be highly irregular; part of oogonial stalk incorporated into the supporting hypha. Oospores terminal or intercalary, almost plerotic to aplerotic (av. 21 µm diam); wall 2-3 µm some 4 µm thick. Ooplast spherical, av. 10 µm diam. Hyphal swellings globose, terminal (av. 24 µm) and numerous, mostly thin-walled, some swellings thick-walled up to 2 µm. Antheridia 1-2 cells per oogonium (av. 5x11 µm), monoclinous to be closely monoclinous, cells inflated with crooked-neck, broad apical attachment to oogonium; stalks unbranched and inserted into the oogonial stalk at a distance of up to 25 µm from the oognium.

The *Pythium* RB I isolates shared many characters with *P. ultimum* var. *ultimum* and *P. irregulare* s.s. However, the morphological characteristics could not confirm isolates as either *P. ultimum* var. *ultimum* or *P. irregulare* s.s. The morphological characteristics of the isolates did, however, not correspond with the described morphological characters of *P. iwayamai.* Descriptions for *P. iwayamai* by Iwayamai (1933); Ito & Tokunaga (1935) and Hirane (1960), as reproduced in Van der Plaats-Niterink (1981), are confusing and imprecise and most characters described for this species were not observed in any of the *Pythium* RB I isolates.

Morphological characteristics of all *Pythium* RB II isolates were similar to those of the *Pythium* RB I isolates (Figs. 3 & 4). No sporangia, but appressoria were observed. Zoospores not produced. Mating system homothallic. Oogonia terminal or intercalary,

globose (av. 21 µm diam), wall smooth or highly irregular with mono- to multi-papillate projections on oogonial wall, some papillae form dichotomous branches; part of oogonial stalk incorporated into the supporting hypha. Oospores mostly plerotic (av. 17 µm diam), wall 2-3 µm thick. Ooplast av. 7 µm diam. Antheridia (1-2 per oogonium) strictly monoclinous, cells (av. 7x12 um) crook-necked, inflated, some sessile or closely monoclinous, cells attached broad apical to oogonium. Antheridial stalks unbranched, not enveloping oogonium, originating at a medium to short distance from oogonium (up to ± 26 um). Hyphal swellings terminal and intercalary (av. 21 µm diam), numerous, globose, thin-walled, some with irregular shapes.

As with *Pythium* RB I, the *Pythium* RB II isolates (STE-U 7548, STE-U 7550, and STE-U 7549) also showed characters similar to *P. ultimum* var. *ultimum* and *P. irregulare* s.s., but differed from these species due to the presence of plerotic or almost plerotic oospores, absence of sporangia, branched oogonial wall papillae, absence of hypogenous antheridia and antheridia applied broad apical to oogonia. The characters observed for these isolates did not correspond to those described for *P. canariense* because: i) catenulate pyriform, dumb-bell-shaped, elongated and papillate sporangia were absent ii) antheridial stalks were unbranched; only 1-2 antheridia per oogonium iii) average size of oospores and oogonia was smaller iii) antheridia were strictly monoclinous, not entangling oogonium iv) oospores varied between mainly plerotic to aplerotic v) some oogonia with a highly irregular, papillate oogonial wall vi) antheridial cells were attached broad apical to oogonial wall. Similarly, characters described for *P. violae* (Van der Plaats-Niterink, 1981; Chesters & Hickman, 1944), were not observed in the *Pythium* RB II isolates, except for the presence of sessile to stalked monoclinous antheridia originating close to oogonium.

Morphological differentiation between *Pythium* RB I and RB II was not possible. The dimensions and range of oospores and oogonia overlapped, antheridial cells, mode of attachment, stalk insertion and oogonial wall papillations did not differ between the two sub-clades or groups.

The CBS reference isolates and other reference isolates (CBS 112353, CBS 156.64, CBS 698.83, CBS 697.83, P16712 and CBS 159.64) were mostly sterile and did not produce any oogonia with oospores or sporangia. Only mycelium and a few hyphal swellings were

observed. When isolate CBS 156.64 was analysed by van der Plaats Niterink, the isolate also did not sporulate.

DNA macroarray analyses. The optimal temperature for hybridization of the arrays was 55 °C, since this resulted in a reasonable signal intensity and no cross-hybridization to closely related non-target species (*P. canariense* CBS 112353, *P. iwayamai* CBS 156.64 and *P. violae* CBS 159.64). At a hybridization temperature of 50 °C, high signal intensity was obtained, but some isolates showed slight cross-hybridization to non-target oligonucleotides that will result in false positive signals. When the hybridization temperature was increased to 60 °C, no signal was detected with any of the PCR amplicons. Therefore, a hybridization temperature of 55 °C was selected for all membrane hybridizations. The two *Pythium* RB I isolates hybridized differentially to the two newly designed oligonucleotides (spiwa1 and spiwa2), since isolates STE-U 7555 hybridized well to spiwa2 (Fig. 5a), whereas, isolate STE-U 7551 showed the best hybridization to spiwa1, and only slight hybridization to spiwa2. The three *Pythium* RB II isolates all showed good hybridization to spcan1. For spcan2, isolate STE-U 7549 showed no hybridization, whereas the other two isolates only showed slight hybridization (Fig. 5b).

Pathogenicity towards rooibos, lupin and oats. Levene's variance ratio test revealed that the variance for the data from the two repeat pathogenicity trials was comparable. The analysis was, therefore, conducted on the combined data of the two trials.

The *Pythium* RB I and RB II isolates were considered to be non-pathogenic toward rooibos, oats and lupin. The isolates did not cause a significant reduction in seedling survival ($P = 0.3665$) (Table 3) or seedling length ($P = 0.8408$) (Table 4) relative to the uninoculated control. The only significant interaction that was observed in the analyses was for crops, but this is not important since it included the uninoculated controls, and it therefore only reflects inherent crop difference in percentage survival and seedling lengths (Tables 3, 4).

DISCUSSION

Twelve *Pythium* clade G isolates from rooibos (three nurseries and one native site) in South Africa were characterised with regards to morphological and phylogenetic

characteristics as well as pathogenicity toward rooibos, oats and lupin. Although the isolates were morphologically similar, they formed two distinct phylogenetic groups (*Pythium* RB I and II) with uncertain taxonomic status. Neither *Pythium* RB I or II isolates were pathogenic towards rooibos seedlings or two nursery rotation crops (lupin and oats). Two oligonucleotides were designed that could detect and distinguish *Pythium* RB I and II from each, and from other related clade G species using DNA-macroarray analyses.

DNA macroarray is a powerful molecular tool that can be used for detecting and differentiating *Pythium* species (Tambong *et al.*, 2006; Zhang *et al.*, 2008). This molecular tool is valuable for the detection of a wide range of fungi and oomycetes, including *P. irregulare* and *Pythium* sp. Py26 (Izzo & Mazzola 2009). Other molecular tools, such as real-time PCR that are faster, quantitative and more sensitive than DNA macroarrays, can also be used for the detection of *Pythium* species (Wang *et al.*, 2003; Schroeder *et al.,* 2006; Cullen *et al.,* 2007; Vincelli & Tisserat, 2008; Spies *et al.* 2011c). However, these techniques are limited in the fact that they can only detect a few pathogens in one assay (Zhang *et al.* 2008). The value of the macroarray lies within its ability to detect and differentiate all known *Pythium* species simultaneously (Tambong *et al*., 2006). However, as more knowledge is gained, the number of *Pythium* species increases and the development and validation of new oligonucleotides for new taxons are required. In the current study, oligonucleotides were designed and conditions were optimized for detecting and differentiating *Pythium* RB I and RB II isolates, which may represent new taxons, from each other. Although two oligonucleotides were designed for each group, only one of the oligonucleotides from each group (spiwa2 and spcan1) hybridized to the tested isolates within each group without cross hybridizing to non-target oligonucleotides. Evaluating different hybridization temperatures was important to prevent cross-hybridization between the two rooibos groups and ensure specificity, as was also found by Zhang *et al.*, (2007). The two developed oligonucleotides will be useful for inclusion in macroarray analyses of rooibos roots and rhizosphere soil from nurseries in future studies. Although the rooibos isolates are non-pathogenic, knowledge of their occurrence is important, since their presence might be an indication of a less harmful *Pythium* community. For example, when these isolates were co-inoculated with a pathogenic species such as *P. irregulare* or other pathogenic oomycete rooibos species, the survival of lupin and oats seedlings was significantly higher, than when only pathogenic oomycete species were inoculated (Chapter 3).

There is substantial diversity within clade G taxa with regards to pathogenicity and their geographic and climatic distribution. The *Pythium* RB I and II rooibos isolates were all non-pathogenic towards three crops, i.e. rooibos, lupin and oats. In contrast, several other clade G species (*P. iwayamai, P. okanoganense,* and *P. paddicum*) are known as snow mold fungi that are pathogenic towards monocot grasses (Iwayama, 1993; Lipps, 1980 a, b; Van der Plaats-Niterink, 1981; Bridge *et al*., 2008). Davison *et al.* (2003) reported *P. violae* as pathogenic towards carrots (*Daucus Carota* L.), causing cavity spot of carrot. They did, however, not investigate the phylogenetic position of their isolates (Davison *et al.,* 2003), and it is thus not known whether their isolates were from clade G, F or I, since *Pythium* isolates with the morphology of *P. violae* can fit into any of these clades (Lévesque & De Cock, 2004; McLeod *et al.*, 2009). *Pythium nagaii* have been reported to cause root rot of rice (*Oryza sativa* L.) seedlings (Van der Plaats-Niterink, 1981). The pathogenicity of *P. canariense* is unknown. Clade G taxa have now been reported from several countries, including Spain, UK, Antarctica, Australia, The Netherlands, South Africa, Japan and the USA. (Lévesque & De Cock, 2004, Paul, 2002; Bridge *et al*., 2008). The rooibos isolates were obtained from the Cederberg Mountains in the Western Cape that is known for its hot dry summers, and poor soils in which rooibos grows. Similarly, the other clade G isolate (*P.* sp. WB-1, PPRI8300) from South Africa was isolated from a region with a very hot climate, the Northern Cape Province, which has a semi-arid to desert climate (McLeod *et al.*, 2009). In contrast, several of the other clade G species (*P. iwayamai, P. okanoganense* and *P. paddicum*) are cold-adapted mesophiles (Bridge *et al.*, 2008). Altogether this suggests that species from this clade are mesophiles that can withstand high and/or low temperature extremes.

The species status of *Pythium* RB I and RB II is uncertain, and they are thus not described here as new species but are referred to as putative new species. A species would be defined as distinct based on a comparison with its closest relatives. Based on our multigene phylogeny, the *Pythium* RB I taxon could be considered as a phylogenetic species, since it is distinct from *P. iwayamai* (CBS 156.64), it closest relative, within sub-clade 1a. However, this closets relative is only represented by one sequence (no other sequences available in GenBank). Therefore, a lack of knowledge exists on the intraspecific variation within *P. iwayamai* as represented by CBS 156.64, and whether the *Pythium* RB I taxon would remain distinct if more isolates similar to CBS 156.64 or *Pythium* RB I would be included. Another complication is the fact that *P. iwayamai* isolate CBS 156.64 is not the type strain of *P.*

iwayamai, but it is the strain used by Van der Plaats-Niterink (1981) in her monograph of the genus. The type strain is no longer available. Isolate CBS 156.64 has not produced oogonia or sporangia since before 1981 when it was used by Van der Plaats-Niterink, creating uncertainty as to the identity of the strain. The original description of *P. iwayamai* (Iwayama, 1933) is confusing and imprecise and is difficult to use in morphological comparisons across species. These uncertainties make it hard to pinpoint the differences between *Pythium* RB I and CBS 156.64, thereby complicating species delineation.

The description of the *Pythium* RB II as a new species is confounded by similar problems than with *Pythium* RB I. The closest relatives of *Pythium* RB I are *P. canariense* and *P. violae*, each represented by only one sequence. *Pythium violae* needs revision since this species is distributed across at least three *Pythium* clades (F, G and I) (McLeod *et al.*, 2009). This is due to several isolates having the morphology of *P. violae*, but the isolates being genetically distinct (Lévesque & De Cock, 2004). The other relative of *Pythium* RB II, *P. canariense* was described using only one isolate (Paul, 2002). Unlike the *Pythium* RB I isolates, the *Pythium* RB II isolates and other isolates in sub-clade 1b showed genetic diversity as was evident by different branch lengths in the ITS and multigene phylogenies, suggesting the presence of several taxa. These taxa can only be defined with certainty if more representatives of sub-clade 1b are included in the phylogenies. The importance of a multigene phylogeny was evident for sub-clade 1b, since in the ITS phylogeny, isolate *P.* aff. *canariense* OW1707 clustered with the *Pythium* RB II isolates, whereas in the combined phylogeny it clustered distinct from *Pythium* RB II suggesting that it could also be a distinct taxon. Unfortunately isolate OW1707 died in storage and could not be studied morphologically. The *Pythium* RB I and II taxa were indistinguishable from each other based on morphology, precluding the use of the morphological species concept for clade G, should these isolates be described in future as new species.

In conclusion, this study characterised two distinct clade G groups (*Pythium* RB I and RB II) that are associated with, but non-pathogenic towards rooibos in South Africa. Despite the distinct nature of these groups in a multi-gene phylogeny, their taxonomic status is uncertain due to ill-described and/or ill-represented closest relatives in clade G. Additionally, the ITS phylogeny indicated at least two other un-described taxa (sub-clades 2 and 3) that also require accurate descriptions using multi-gene data and morphological characteristics. The taxon status of these species and the entire clade G is in dire need of a more in depth

study and re-description, and a concerted effort should be made by all researchers having access to representatives of clade G. Ideally sporulating representatives with very high molecular similarity to non-sporulating and/or ill-described clade G ex-type strains (or authoritative strains, such as those used in the monograph of Van der Plaats-Niterink [1981]) would need to be found in order to epitipify such species and clear up their relationship to putative new taxa such as *Pythium* RB I and RB II.

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Table 1. Origin and morphological- and phylogenetic identities of *Pythium* isolates obtained from rooibos in South Africa.

Isolate [a]	Genbank accession[b]				Nursery/ Native population	Isolation date	Morphological identification	Rooibos group based on phylogeny[c]
	ITS	*COX1*	*COX2*	β-tubulin				
STE-U 7548	JQ412771	JQ412796	JQ412808	JQ412784	B	2008	*P. irregulare* s.s./*P. ultimum* var. *ultimum*	II
STE-U 7549	JQ412770	JQ412795	JQ412807	JQ412783	C	2008	*P. irregulare* s.s./*P. ultimum* var. *ultimum*	II
STE-U 7550	JQ412777	JQ412801	JQ412813	JQ412789	N	2009	*P. irregulare* s.s./*P. ultimum* var. *ultimum*	II
STE-U 7551	JQ412768	JQ412793	JQ412805	JQ412781	N	2007	*P. irregulare* s.s./*P. ultimum* var. *ultimum*	I
STE-U 7552	JQ412769	JQ412794	JQ412806	JQ412782	N	2007	*P. irregulare* s.s./*P. ultimum* var. *ultimum*	I
STE-U 7553	JQ412767	JQ412792	JQ412804	JQ412780	N	2007	*P. irregulare* s.s./*P. ultimum* var. *ultimum*	I
STE-U 7555	JQ412772	JQ412797	JQ412809	JQ412785	Native population	2009	*P. irregulare* s.s./*P. ultimum* var. *ultimum*	I
STE-U 7556	JQ412773	JQ412798	JQ412810	JQ412786	Native population	2009	*P. irregulare* s.s./*P. ultimum* var. *ultimum*	I
STE-U 7557	JQ412779	JQ412803	JQ412815	JQ4127791	Native population	2009	*P. irregulare* s.s./*P.*	I

Isolate [a]	Genbank accession[b]				Nursery/ Native population	Isolation date	Morphological identification	Rooibos group based on phylogeny[c]
	ITS	*COX1*	*COX2*	β-tubulin				
							ultimum var. *ultimum*	
STE-U 7558	JQ412774-75	JQ412799	JQ412811	JQ412787	Native population	2009	*P. irregulare* s.s./*P. ultimum* var. *ultimum*	I
STE-U 7559	JQ412778	JQ412802	JQ412814	JQ412790	Native population	2009	*P. irregulare* s.s./*P. ultimum* var. *ultimum*	I
STE-U 7560	JQ412776	JQ412800	JQ412812	JQ412788	Native population	2009	*P. irregulare* s.s./*P. ultimum* var. *ultimum*	I

[a] Culture collection number of the Stellenbosch University culture collection.

[b] GenBank accession numbers of the internal transcribed spacers (ITS) sequence, cytochrome oxidase subunit (*COX* 1 and *COX*2) and β-tubulin of each isolate.

[c] Phylogenetic analyses of the ITS region and a multiple gene phylogeny identified two major groups to which the rooibos isolates belonged, i.e. *Pythium* RB I and II.

Table 2. Oligonucleotides that were designed to identify two rooibos group G *Pythium* isolates, *Pythium* RB I and II.

Oligonucleotide	Rooibos Group	Sequence
spiwa1	I	GTTATCCACTTTGCAGTGGAGTGAC
spiwa2	I	GGTTGGTGAAGTGTTGTCTC
spcan1	II	GCCTGTGCGGTCGACATG
spcan2	II	GGTTGGTGCTGTGTGTGTTGTC

Table 3. Analyses of variance for the effect of *Pythium* clade G isolates from rooibos, which represented two groups (*Pythium* RB I and II), on mean percentage survival of rooibos, lupin, and oats.

Source of variation	DF	MS	SL
Rep(Trial)	5	130.19841	0.0451
Species (control included)	2	157.96561	0.0615
Group (control included)	3	106.80423	0.1282
Isolates (control included)	6	60.71693	0.3665
Crop	2	9363.43651	<0.0001
Species × Crop	4	106.56878	0.1105
Group x Crop	6	75.50265	0.2339
Isolate × Crop	12	89.51058	0.0966
Experimental error	100	55.09175	
Corrected total	125		

Table 4. Analyses of variance for the effect of *Pythium* clade G isolates, which represented two groups (*Pythium* RB I and II), from rooibos on mean seedling length of rooibos, lupin, and oats.

Source of variation	DF	MS	SL
Rep(Trial)	5	1122.563	0.0635
Species	2	270.435	0.5946
Group	3	342.968	0.5769
Isolate	6	234.855	0.8408
Crop	2	1337790.260	<0.0001
Species × Crop	4	747.707	0.2249
Group x Crop	6	1072.561	0.0631
Isolate × Crop	12	903.376	0.0682
Experimental error	100	517.554	
Sample error	1134	231.429	

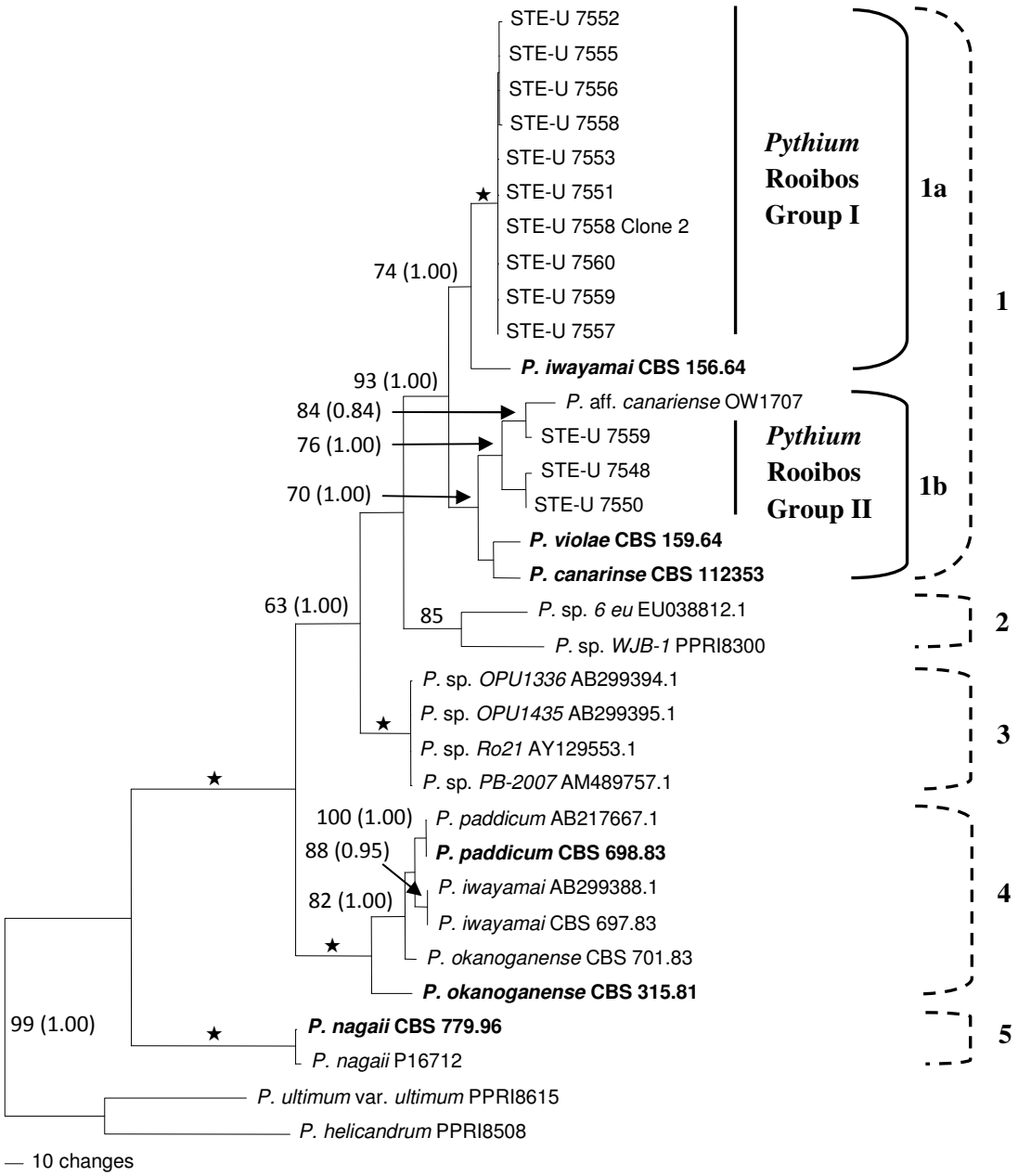

Fig 1. Phylogeny of *Pythium* clade G (Lévesque & De Cock 2004) based on the internal transcribed spacer region of nuclear rDNA. The tree is one of 723 equally parsimonious trees of a heuristic search. The ex-type, ex-holotype strains or strains used by van der Plaats-Niterink (1981) are indicated in bold. Numbers in the tree represent parsimony bootstrap support values followed by posterior probabilities (in brackets). Sub-clade numbers (1 – 5) are indicated to the right of the tree. Bootstrap support of 100 % and a probability value of 1.00 are indicated by a star symbol (⋆). TL = 957; CI = 0.687; RI = 0.822; RC = 0.565; HI = 0.313.

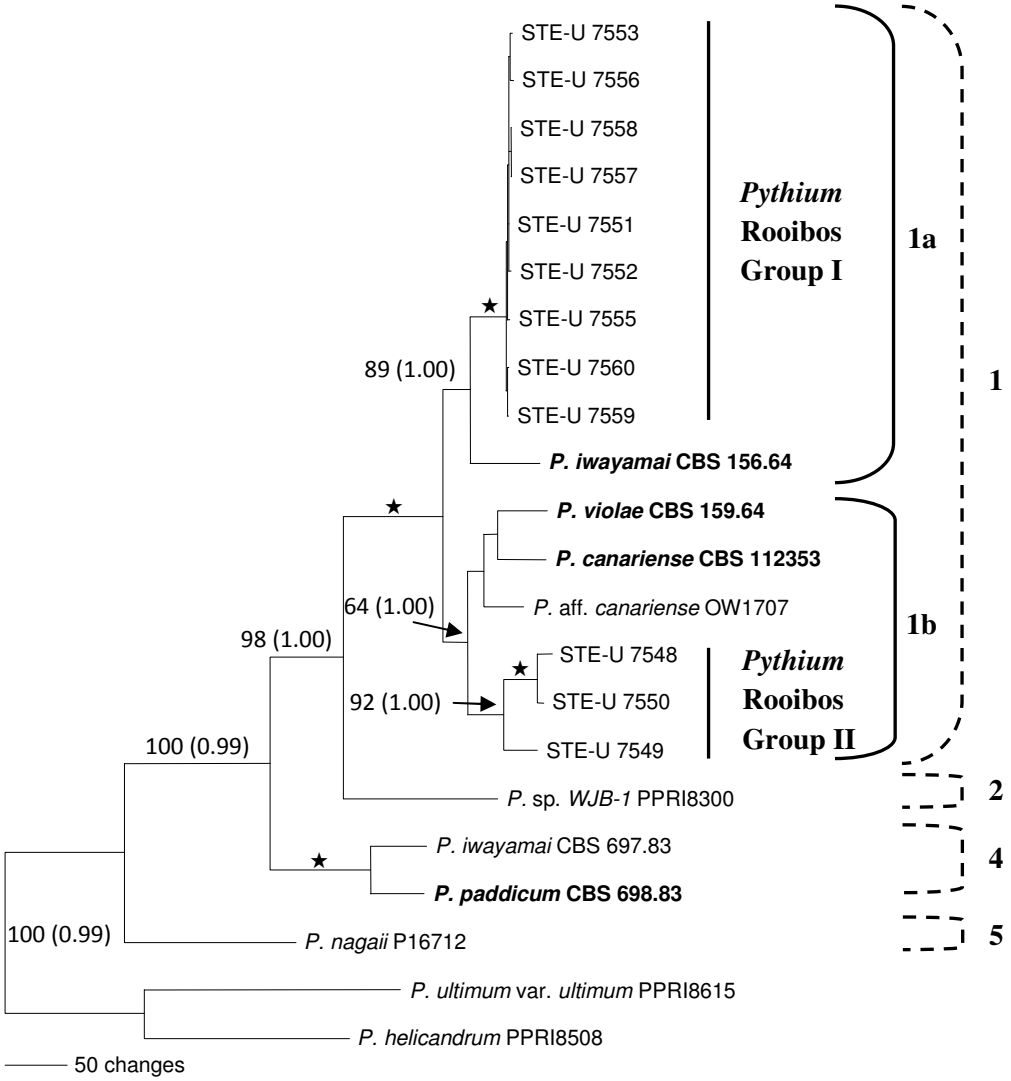

Fig 2. Phylogeny of Pythium clade G (Lévesque & De Cock 2004) based on the combined ITS, β-tubulin and cytochrome *c* oxidase subunit (*COX*) *1*, *COX2* regions of nuclear rDNA. The tree is one of 239 equally parsimonious trees of a heuristic search. The ex-holotype strains or strains used by van der Plaats-Niterink (1981) are indicated in bold. Numbers in the tree represent parsimony bootstrap support values followed by posterior probabilities (in brackets). Bootstrap support of 100 % and a probability value of 1.00 are indicated by a star symbol (⋆). The sub-clade numbers (1, 2, 4 and 5) indicated to the right of the tree, are according to sub-clades identified in an ITS phylogeny (see Fig. 1). TL = 1640; CI = 0.707; RI = 0.676; RC = 0.478; HI = 0.293.

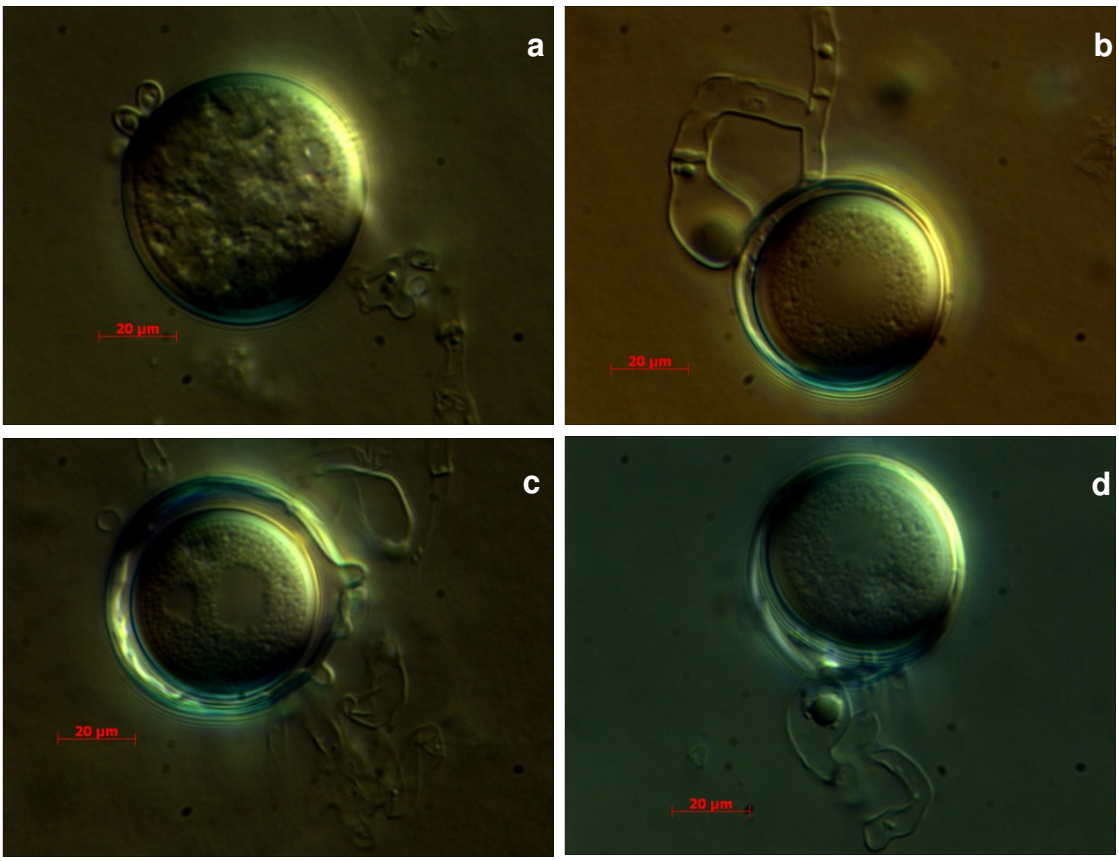

Fig. 3. . Sexual structure of *Pythium* Rooibos Group I isolates. (a) plerotic oospore; (b-d) various contacts of antheridia with oogonia.

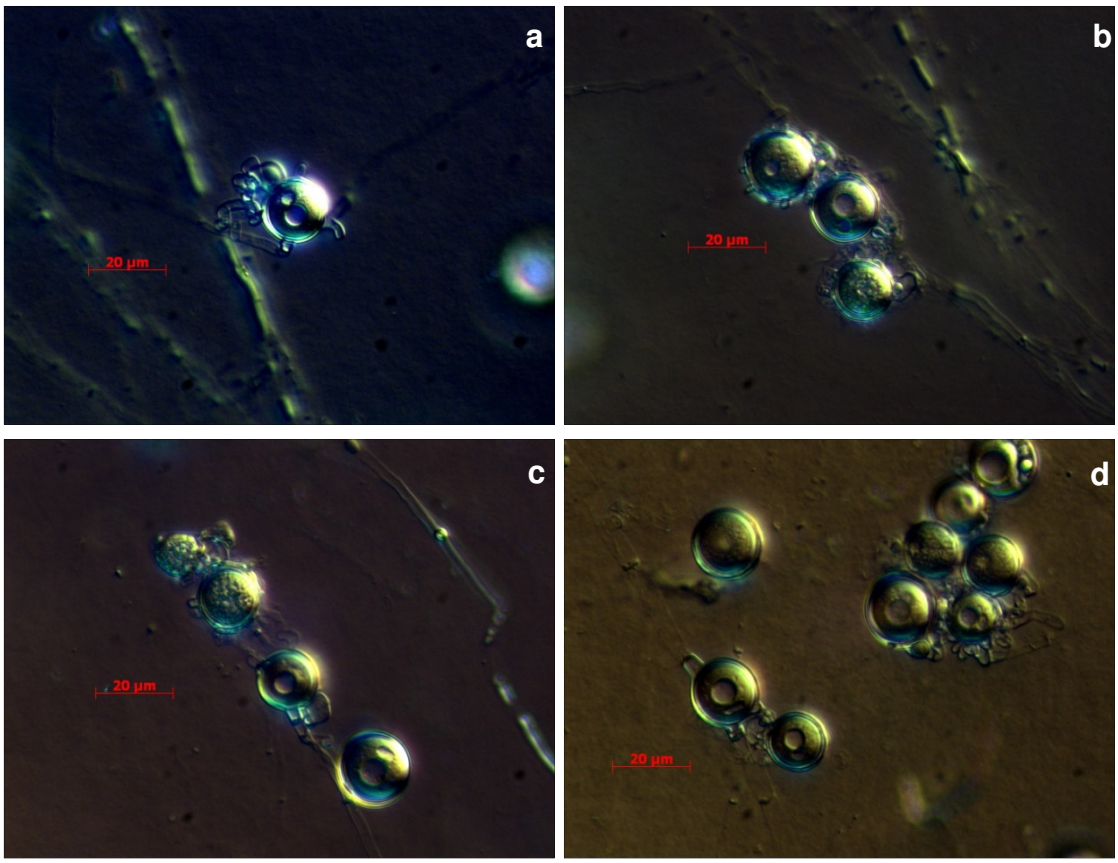

Fig. 4. Sexual structures of *Pythium* Rooibos Group II isolates. (a-d) antheridia and oogonial contact.

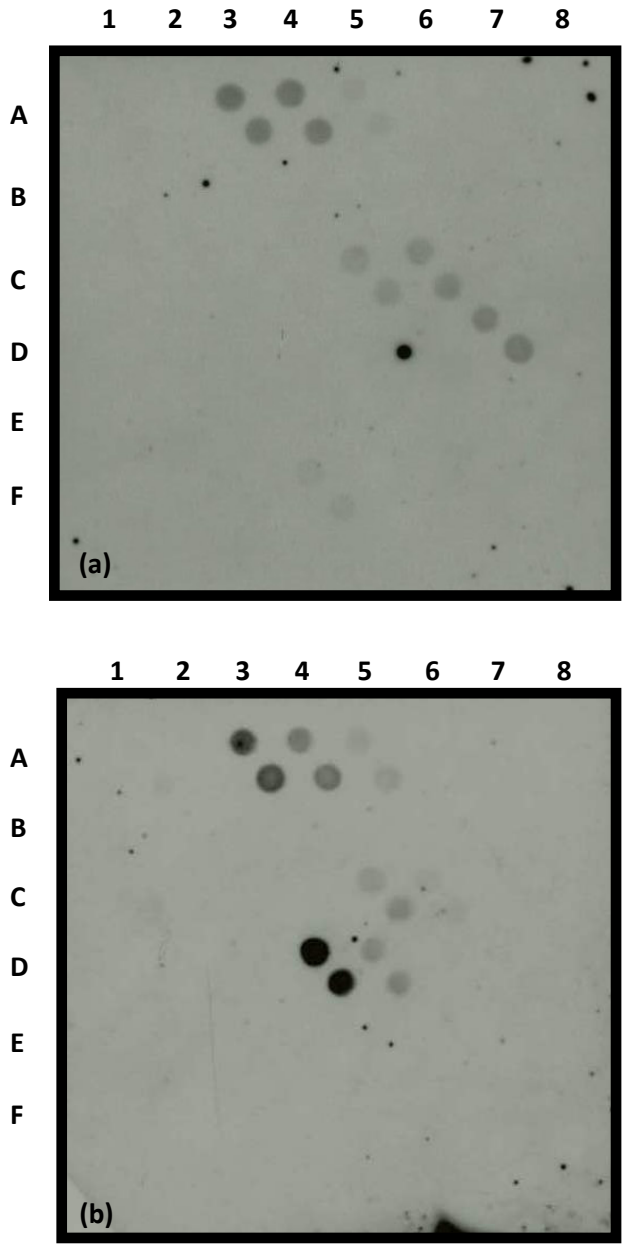

Fig. 5. Chemiluminograms showing hybridization patterns of dioxigenin-labeled amplicons obtained after PCR amplification of DNA of pure cultures of a (a) *Pythium* rooibos group I isolate (STE-U 7555) and (b) *Pythium* rooibos group II isolate (STE-U 7550). The oligonucleotides were spotted in duplicate, hybridized overnight and exposed on Kodak X-ray film for 24 h. Pairs of diagonally arranged black to grey dark spots indicate positive hybridization signals. The spots that hybridized are A3 to A5, genus-specific oligonucleotides; C5 and C6, iwa51 and iwa52, respectively; D4 to D7, spcan 1, spcan2, spiwa1, spiwa2, respectively; F4, vioIwa. Chemiluminograms were conducted twice and similar patterns were obtained.

3. *PYTHIUM* SPECIES ASSOCIATED WITH ROOIBOS SEEDLINGS, AND THEIR PATHOGENICITY TOWARDS ROOIBOS, LUPIN AND OATS

ABSTRACT

Rooibos (*Aspalathus linearis*) is an important indigenous crop that is only grown in a small area in the Western Cape Province of South Africa. Although oomycetes, mainly *Pythium,* are a common problem that causes serious losses in rooibos nurseries, limited information is available on the specific species involved. Only two non-pathogenic *Pythium* clade G groups (*Pythium* RB I and RB II) are known to be associated with rooibos. The aims of this study were to investigate (i) oomycete species composition in 19 rooibos nurseries, (ii) species composition in a native rooibos ecosystem, (iii) pathogenicity of the identified species towards rooibos and two rotation crops (lupin and oats) and (iv) whether non-pathogenic *Pythium* species can suppress damping-off caused by pathogenic oomycete species on rooibos, lupin and oats. Molecular and morphological analyses of 118 oomycete isolates from nurseries and 33 isolates from the native site, revealed the presence of several *Pythium* species including *P. acanthicum, P. irregulare* (five molecular groups), *P. mamillatum, P. myriotylum*, *P. pyrilobum*, *Pythium* RB I and *Pythium* RB II (two molecular groups) and one isolate of *Phytophthora cinnamomi.* Most of the species were identified in nurseries and native rooibos. The exceptions were the restriction of *P. acanthicum, P. myriotylum* and *Pythium* RB II to nurseries, and *Ph. cinnamomi* and *P. pyrilobum* to native rooibos. In nurseries, *P. irregulare* was the most common species (77%) followed by *P. myriotylum* (14%). *Pythium irregulare* was also the most prevalent species (45%) in native rooibos, but *P. pyrilobum* (21%) was second most prevalent. All species, except *P. acanthicum* and the previously characterised *Pythium* RB I and RB II, were pathogenic and highly virulent towards rooibos causing 100% damping-off. On lupin, *P. acanthicum* and *Pythium* RB I and RB II were also the only non-pathogenic species, with *Ph. cinnamomi* and *P. pyrilobum* being among the most virulent species, but they were less virulent than on rooibos. On oats, only *P. irregulare, P. myriotylum* and *P. pyrilobum* were pathogenic. Not all *Pythium* isolates within a species were pathogenic, since some non-pathogenic *P. myriotylum* isolates were identified on oats and lupin, *P. mamillatum* isolates on lupin, and *P. irregulare* isolates on oats. The co-inoculation of pathogenic and non-pathogenic *Pythium* species only resulted in significant disease suppression in the less susceptible crops (lupin and oats), but not on rooibos.

INTRODUCTION

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 (Joubert *et al.*, 2008). Special production practices are required for cultivating rooibos, since it is difficult to establish and grow. One of the main factors that affects establishment is damping-off, which occurs within the first 6 weeks of rooibos seedling growth. Damping-off can cause serious losses in nurseries and can be caused by several plant pathogens including *Pythium, Fusarium* and *Rhizoctonia* spp. Of these, pathogens within the genus *Pythium* are among the most prevalent and important pathogens (Personal communication, S.C. Lamprecht, ARC-Plant Protection Research Institute, South Africa).

The genus *Pythium* belongs to the phylum Oomycota, commonly known as oomycetes, and consists of more than 120 described species that include several soilborne pathogens. Another oomycete genus that includes important soilborne pathogens is *Phytophthora* (Alexopoulos *et al.*, 1996; Dick, 2001). *Pythium* species are very common in soils and have been reported from all over the world (Van der Plaats-Niterink, 1981; Dick, 1990). Many *Pythium* 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 (Chellemi *et al.*, 2000; Zhang & Yang, 2000; Higginbotham *et al.*, 2004; Matoba *et al.*, 2008). However, several species are saprophytic with some species even encouraging plant growth and having potential as biocontrol agents (Martin & Loper, 1999; Van der Plaats-Niterink, 1981). For example, *P. acanthicum* Drechsler, *P. oligandrum* Drechsler and *P. periplocum* Drechsler are effective at suppressing damping-off caused by *P. ultimum* Trow on sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) (Martin & Hancock, 1987) and on cucumber (*Cucumis sativus* L.) (Ali-Shtayeh & Saleh, 1999). These biocontrol species have also shown potential for suppressing *P. irregulare* Buiman, *P. mamillatum* Meurs, *P. myriotylum* Drechsler and *P. vexans* de Bary (Van der Plaats-Niterink, 1981; Ribeiro & Butler, 1995). *Pythium oligandrum* has a very wide biological control range, since it is also antagonistic towards *Phytophthora parasitica* Dastur (Picard *et al.*, 2000), *Rhizoctonia solani* Kühn and *Fusarium culmorum* (W.G. Smith) Sacc. (Deacon, 1976). On apple (*Malus domestica* Borkh.), several un-described *Pythium* species (*Pythium* MM1, *Pythium* MM3 [*aff. oedochilum*] and *Pythium* MM5 [*aff. vexans*]) have shown biocontrol potential, since they

were able to suppress root rot on apple seedlings caused by *P. sylvaticum* Campbell & Hendrix and *P. ultimum* (Mazzola *et al.*, 2002).

The management of *Pythium* diseases requires an integrated approach, where several cultural practices, biological control and fungicides can form part of an integrated management strategy. In South Africa, several fungicides (metalaxyl, metalaxyl-M, mancozeb, thiram, captab, dazomet and dichlorophen) are registered for the management of *Pythium* diseases (Nel *et al.*, 2003), of which metalaxyl is most effective (Erwin & Ribeiro, 1996). Fungicides may, however, not be applied in organic production systems and therefore only biological control and cultural practices (water management, fertilizer applications, organic amendments and crop rotation) can be used (Ko & Kao, 1989; Erwin & Ribeiro, 1996; Hoitink *et al.*, 1997; Mazzola & Gu, 2000; Naseby *et al.*, 2000). Excess water and soil moisture favours the reproduction and infection of *Pythium* and other oomycetes. Therefore water management is very important in the management of these pathogens (Gubler *et al.*, 2004). Rotation crops and organic amendments can sometimes be effective at suppressing soilborne diseases, such as *Pythium* (Fry, 1982; Davison & McKay, 2003; Hoitink & Changa, 2004). The mechanisms of suppression might be due to the release of fungitoxic compounds or/and an improvement in general microbial activity (Hoitink & Boehm, 1999; Gu & Mazzola, 2003; Larkin & Griffin, 2007; Wang *et al*., 2009). Biological control has also shown potential for the management of *Pythium* damping-off in some crop systems (Hadar *et al.*, 1984; Loper, 1988; Whips & Lumsden, 1991; Naseby *et al.*, 2000; Nelson *et al.*, 2004; Quagliotto *et al.*, 2009). The only biocontrol agent that is registered for *Pythium* root rots in South Africa is *Trichoderma harzianum* Rifai (personal communication, N. Mkize, Department of Agriculture, Forestry and Fisheries, directorate Food Safety and Quality Assurance, South Africa).

Identification of *Pythium* species can be conducted using morphological and molecular methods. Traditionally, morphological characteristics have been used for species identification, but this is difficult and requires a substantial amount of experience (Martin 2000). Therefore, molecular species identification using the internal transcribed spacer regions (ITS) has become very popular for identifying species, using either Polymerase Chain Reaction Restriction Fragment Length polymorphisms (PCR-RFLP) or sequence data (Lévesque & De Cock, 2004; Mazzola *et al.*, 2009). The ITS regions have also been used to identify 11 phylogentic clades (A to K) among the known *Pythium* species (Lévesque & De Cock, 2004). For some of the *Pythium* clades, the validity of several species within the clade

has been questioned. For example, in clade F, Garzón *et al.* (2007) identified two distinct species, *P. cryptoirregulare* and *P. irregulare* s.s. within the *P. irregulare* species complex. However, Spies *et al.* (2011a) provided evidence that all currently described species within the *P. irregulare* complex (*P. cryptoirregulare*, *P. cylindrosporum, P. irregulare* and *P. regulare*) might be comprised of a single phylogenetic species that is genetically diverse.

Very little is known about the specific *Pythium* species and other oomycetes associated with rooibos, and their pathogenicity towards rooibos, and two nursery rotation crops (lupin [*Lupinus angustifolius* L.] and oats [*Avena sativa* L.]). Previously, only two *Pythium* clade G groups (*Pythium* RB I and RB II) were identified from rooibos, which were shown to be non-pathogenic towards rooibos, lupin and oats. The species status of these isolates is uncertain, but they may represent two or more new species based on phylogenetic data (Chapter 2). Knowledge of the specific oomycete species involved is important, since 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. Therefore, the aims of this study were to determine (i) the oomycete species associated with rooibos seedlings in 19 nurseries, (ii) if the species in a native rooibos ecosystem (mature plants in native ecosystem) are similar to those in nurseries, (iii) the pathogenicity of oomycete species towards rooibos and two rotation crops (lupin and oats) and (iv) whether co-inoculation of pathogenic and non-pathogenic oomycete species will influence the level of disease development on rooibos, lupin and oats.

MATERIALS AND METHODS

Survey and isolations from rooibos nurseries and native ecosystem. Rhizosphere soils from 19 rooibos nurseries were sampled from a depth of 20 cm in March/April and in June/July 2007 to 2009. Rhizosphere soil was also collected from rooibos plants in a native ecosystem (Table 1). The soil was used in a glasshouse trial, where rooibos seeds were sown into the different soils, and kept at approximately 18 °C night and 27 °C day temperatures. After 4 weeks, the roots of damped-off seedlings were plated out onto *Pythium* and *Phytophthora* selective media, PARP and PARPH respectively (Jeffers & Martin, 1986; Chellemi *et al.*, 2000). Hyphae emerging from the plated roots were hyphal tipped to corn meal agar (CMA, Sigma Aldrich, St Louis, USA), followed by a second hyphal tipping. All isolates were stored as CMA culture plugs in sterile de-ionised water containing grass blades,

as V8-agar (Galindo & Gallegly, 1960) plugs in sterile de-ionised water, as well as potato-carrot agar (Dhingra & Sinclair, 1985) slant cultures at 15 °C.

Molecular species identification. Oomycete isolates were grown on V8 agar for 5-7 days. Aerial mycelium was harvested and genomic DNA was extracted from a total of 151 isolates using a slight modification of the CTAB method of Lee and Taylor (1990) (Tewoldemedhin *et al.*, 2011a).

Polymerase chain reaction Restriction Fragment Length polymorphisms (PCR-RFLPs) analyses. The ITS region of the isolates was amplified from DNA using primers ITS6 (Cooke & Duncan, 1997) and ITS4 (White *et al.*, 1990). The PCR reaction consisted of 1× PCR buffer (Bioline, London, United Kingdom), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.65 U BIOTAQ™ (Bioline), 0.05 mg/ml bovine serum albumin (BSA) Fraction V (Roche Diagnostics South Africa, Randburg, South Africa) and 0.2 mM of each primer in a total reaction volume of 40 µl. Amplications were performed in a 2720 Applied Biosystems (Foster City, CA) 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 a final 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 (Mazzola *et al.*, 2009), according to manufacturer's instructions (Fermentas Inc., Glen Burnie, Maryland, USA). 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 region of at least two isolates of each PCR-RFLP group was sequenced as previously described (McLeod *et al.*, 2009). The sequences were submitted to BLAST analyses in GenBank, and identified to species level using sequences submitted by Lévesque and De Cock (2004) and Bahramisharif (Chapter 2).

Morphological identification of oomycete species. Morphological identifications of all *Pythium* isolates were conducted as previously described (McLeod *et al.*, 2009). *Phytophthora* species were identified using Erwin and Ribeiro (1996).

Pathogenicity assay of oomycete species. A subset of 32 isolates was selected for inclusion in pathogenicity assays on lupin, oats and rooibos. The isolates represented all the

different species that were identified from nurseries and the native ecosystem (see Results and Table 1), except for *Pythium* RB I and RB II that were not included in the pathogenicity studies, since the pathogenicity of these groups has previously been established (Chapter 2). The selected isolates included one *Phytophthora cinnamomi* isolate, two isolates of *P. acanthicum,* 16 isolates of *P. irregulare,* three *P. mamillatum* isolates, six isolates of *P. myriotylum* and four isolates of *P. pyrilobum.*

Pathogenicity assays were conducted in a glasshouse in pots. A pasteurized planting medium (equal parts of soil, perlite, and sand) was inoculated separately with sand and wheat bran inoculum of each oomycete isolate at a concentration of 0.05% inoculum/planting medium (wet wt/wet wt). The sand-wheat bran inoculum was prepared as previously described (Lamprecht, 1986; Chapter 2). The control consisted of planting medium inoculated with sand-wheat bran that was inoculated with un-colonized agar plugs. Plastic pots (13 cm diameter) were filled with 800g of the inoculated media. Ten planting holes for seeds were made in each pot to a depth of 1.5 cm (rooibos) to 2 cm (lupin and oats). The number of seeds planted per pot was 50 for rooibos and oats, and 20 seeds for lupin. Three replications were included for each treatment, and the trial was conducted twice. The experiments were carried out in the glasshouse at approximately 18 °C night and 27 °C day temperatures.

Pathogenicity of the isolates was determined by evaluating seedling length and percentage survival. The survival of seedlings in all three crops was evaluated two weeks after planting. Seedling length was measured for lupin and oats two weeks after planting, but for rooibos it was only evaluated after one month, since rooibos is a very slow grower and only cotyledons are present two weeks after planting. Re-isolations were made from the seedlings to fulfill Koch's postulates (Tewoldemedhin *et al.*, 2011a).

Evaluating the potential of non-pathogenic *Pythium* species to suppress damping-off caused by pathogenic oomycete species. The ability of non-pathogenic *Pythium* species to suppress damping-off caused by pathogenic oomycete species was assessed on rooibos, lupin and oats 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), and in a previous study (Chapter 2). Each species was represented by only one isolate and included *Ph. cinnamomi* (R8303A), *P. acanthicum* (R8206Q), *P. irregulare* (R8308F), *P. mamillatum* (R8201C), *P. myriotylum* (R8197F), *P. pyrilobum* (R8198A) and *Pythium* RB I (STE-U 7555) and II (STE-U 7548). The isolates that were used to represent

pathogenic species were all pathogenic towards lupin and rooibos, whereas, only *P. irregulare* (R8308F), *P. myriotylum* (R8197F) and *P. pyrilobum* (R8298A) were pathogenic towards oats (see Results section). In each mixed species inoculation treatment (M1 to M8), 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% (w/w). The trial included eight treatments and three replicates per treatment, and was conducted twice. The eight treatments and the specific species used in the co-inoculations consisted of: M1, pathogenic oomycetes spp. (*Ph. cinnamomi, P. irregulare, P. mamillatum, P. myriotylum* and *P. pyrilobum*) and non-pathogenic *Pythium* spp. (*P. acanthicum*, *Pythium* RB I and II); M2, pathogenic oomycetes spp. (*Ph. cinnamomi, P. irregulare, P. mamillatum, P. myriotylum* and *P. pyrilobum*); M3, non-pathogenic *Pythium* spp. (*P. acanthicum, Pythium* RB I and II); M4, *P. irregulare* and non-pathogenic *Pythium* spp. (*P. acanthicum, Pythium* RB I and II); M5, *Ph. cinnamomi* and non-pathogenic *Pythium* spp. (*P. acanthicum, Pythium* RB I and II); M6, native rooibos oomycete species including the pathogenic oomycete spp. (*Ph. cinnamomi, P. irregulare, P. mamillatum* and *P. pyrilobum*) and one non-pathogenic *Pythium* sp. (*Pythium* RB I); M7, *P. irregulare*; M8, *Ph. cinnamomi*. The control for co-inoculation studies consisted of un-colonized sand-bran medium inoculated at 0.05% (w/w) into the planting medium.

Statistical analyses. Statistical analyses were performed for both trials. Levene's test was conducted for homogeneity of trials (Levene, 1960) and the data were subjected to analysis of variance using SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC, USA). The Shapiro-Wilk test was done to test for normality (Shapiro & Wilk, 1965) and Fisher's least significant difference (LSD) for each of these parameters (Ott, 1998) was also calculated.

RESULTS

Survey and isolations from rooibos nurseries and native ecosystem. Oomycete isolates, mainly *Pythium*, were obtained from all the surveyed nurseries and from native rooibos soils. The only other oomycete genus that was identified was *Phytophthora,* which was represented by one isolate from native rooibos. In total 151 oomycete isolates, 118 from nurseries and 33 from the native ecosystem, were obtained and characterised further.

Molecular and morphological identification of oomycete species. ITS-PCR-RFLP analyses revealed the presence of 13 different groups (PY1 to PY13) (Table 1; Fig 1). GenBank BLAST analyses of the ITS sequences of representative isolates of each of the PCR-RFLP groups showed that *P. irregulare* was represented by five PCR-RFLP groups (PY1, 2, 4, 5 and 8), PY3 was *P. myriotylum,* PY6 was *P. mamillatum,* PY7 was *P. pyrilobum,* PY9 was *Ph. cinnamomi*, PY10 was *P. acanthicum, Pythium* RB I was PY 11 and *Pythium* RB II was represented by two groups (PY 12 and 13). For the PCR-RFLP groups of *P. irregulare,* PY4 (32%) and PY2 (21%) was most abundant, followed by PY5 (12%), PY1 (7%) and PY8 (3%) (Table 1). Morphological analyses of all the oomycete isolates corresponded to the molecular identifications.

In nurseries, four *Pythium* species were identified along with the two previously identified *Pythium* clade G groups (*Pythium* RB I and RB II). *Pythium irregulare* was isolated from all the nurseries and dominated populations, comprising 77% (91/118) of all nursery isolates (Table 1). The next most abundant species was *P. myriotylum* (14%), followed by *P. mamillatum* (3%), *Pythium* RB I (3%) and II (3%), and *P. acanthicum* (2%). In contrast to native rooibos where *P. pyrilobum* was prevalent, this species and *Ph. cinnamomi* was 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. In nursery N several species were identified including *P. irregulare, P. myriotylum, Pythium* RB I and RB II. Nursery I contained *P. acanthicum, P. irregulare, P. mamillatum* and *P. myriotylum*. In both nurseries *P. myriotylum* was the dominating (50%) species. *Pythium* populations in nursery K consisted of *P. irregulare* and *P. myriotylum*, which were also dominated by *P. myriotylum*. *Pythium 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* species detected. Furthermore, in all nurseries where more than two *P. irregulare* isolates were obtained, at least two different *P. irregulare* PCR-RFLP groups were identified. Nursery H was the exception, since all five isolates belonged to the same PCR-RFLP group. In contrast, nursery D contained *P. irregulare* isolates that were representative of all five *P. irregulare* PCR-RFLP groups (Table 1).

Among the 33 oomycete isolates obtained from native rooibos soils, *P. irregulare* was also the dominant (45%) species, followed by *P. pyrilobum* (21%), *Pythium* RB I (18%) and *P. mamillatum* (12%). *Phytophthora cinnamomi* (1%) was represented by only one isolate. 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 were not identified from native rooibos (Table 1).

Pathogenicity assay of oomycete species. Analysis of variance based on Levene's variance ratio test indicated that the two repeat trials were comparable and that the data of the two trials could be combined. Significant isolate × crop, species × crop and group × crop interactions were recorded for percentage survival ($P < 0.0001$) and seedling length ($P < 0.0001$) (Tables 2, 3).

Most oomycete isolates and species were pathogenic toward rooibos. All the isolates of *Ph. cinnamomi, P. irregulare, P. mamillatum, P. myriotylum* and *P. pyrilobum* were highly virulent, causing 100% seedling damping-off (Table 4). *Pythium acanthicum* was the only non-pathogenic species, since it did not cause a significant decrease in rooibos seedling survival or seedling length compared to the un-inoculated control (Tables 4, 5).

Several of the *Pythium* species and *Ph. cinnamomi* were pathogenic towards lupin. *Phytophthora cinnamomi, P. irregulare, P. mamillatum, P. myriotylum* and *P. pyrilobum* were all considered pathogenic towards lupin since they significantly reduced the survival of lupin seedlings (Tables 4, 6), and caused a significant reduction in seedling length (except *P. mamillatum*) compared to the un-inoculated control (Tables 5, 7). Similar to rooibos, *P. acanthicum* was also non-pathogenic towards lupin and did not cause a significant effect on the survival or length of lupin seedlings (Tables 4, 5). *Phytophthora cinnamomi* was the most virulent species, followed by *Pythium pyrilobum*, with both species causing significant less seedling survival than *P. irregulare* as a group (Table 6). All the *P. irregulare* isolates were pathogenic towards lupin causing a significant reduction in seedling survival, with the exception of isolate R8304C (PY8), which only caused a significant reduction in seedling length (Tables 4, 5; Fig. 2). The isolates of *P. irregulare* differed in virulence towards lupin, for example isolate R8160L (PY8) caused significant more seedling damping-off than several of the other isolates (Table 4). Not all isolates of *P. mamillatum* and *P. myriotylum* were

pathogenic. Only one of the *P. mamillatum* isolates (R8201C) was pathogenic causing a significant reduction in seedling survival and length compared to the un-inoculated control, whereas the other two isolates did not (Tables 4, 5). Almost all of the *P. myriotylum* isolates were pathogenic, with the exception of isolate R8166K1, which did not significantly affect lupin seedling survival or length (Tables 4, 5). The PCR-RFLP groups of *P. irregulare* differed in virulence from each other, for example, PY2 and PY8 was more virulent than PY4, causing significantly less seedling survival (Table 8), and PY8 also causing a significant reduction in seedling length compared to PY 4 (Table 9). However, it was difficult to generalize for PY groups, since isolates within PY groups also differed significantly from each other in virulence, for example isolates within PY2 (Table 4).

The pathogenicity assays on oats revealed that *P. irregulare, P. myriotylum* and *P. pyrilobum* were pathogenic causing significant less seedling survival (Tables 4, 6), and *P. pyrilobum* also causing a significant reduction in seedling length (Tables 5, 7), when compared to the un-inoculated control. As a group, *P. myriotylum* and *P. pyrilobum* were more virulent than *P. irregulare*, causing significantly less survival of oats seedlings (Table 6). All *Ph. cinnamomi, P. acanthicum* and *P. mamillatum* isolates were non-pathogenic and did not cause a significant reduction in seedling survival or length (Tables 4, 5). Similar to what was found on lupin, isolates within species differed in their pathogenicity. Five of the *P. irregulare* isolates (R8129Q, R8304C, R8307N, R8161C and R8244H) were non-pathogenic towards oats and did not cause a significant reduction in either seedling survival or length. The remaining *P. irregulare* isolates were pathogenic and significantly reduced seedling survival and/or length (Tables 4, 5; Fig 2). Only three (R8169S, R8197F and R8204F) of the six *P. myriotylum* isolates were pathogenic causing a significant reduction in seedling survival (Table 4) and length (except isolate R8169S) compared to the un-inoculated control (Table 5). All isolates of *P. pyrilobum* were pathogenic causing a significant reduction in seedling survival (Table 4) and length (Table 5). When *P. irregulare* isolates were grouped according to PCR-RFLP groups, only PY2 and PY8 were pathogenic, causing a significant reduction in seedling survival and length (only PY8) compared to the uninoculated control (Tables 8, 9).

Evaluating the potential of non-pathogenic *Pythium* species to suppress damping-off caused by pathogenic oomycete species. In co-inoculation pathogenicity assays, Levene's variance ratio test indicated that an analysis of the combined data of the two trial repeats could be conducted. The analyses of variance on the percentage survival and seedling

length showed a significant interaction between molecular group and crop ($P < 0.0001$) (Tables 10, 11).

The co-inoculation of pathogenic (*Ph. cinnamomi, P. irregulare, P. mamillatum, P. myriotylum* and *P. pyrilobum*) and non-pathogenic *Pythium* species (*P. acanthicum, Pythium* RB I and RB II) showed that non-pathogenic species was effective in suppressing disease only in the less susceptible crops lupin and oats, but not on rooibos. On rooibos, the presence of non-pathogenic *Pythium* species along with pathogenic oomycete species still resulted in 100% damping-off of seedlings (Table 13). Similarly, on lupin when several pathogenic oomycete species was present (M1), non-pathogenic species could not significantly suppress damping-off, although seedling length was increased (Tables 12, 13). In contrast, when only *P. irregulare* was inoculated on lupin, the non-pathogenic *Pythium* species (M4) was able to suppress disease and caused a significant increase in seedling survival and length, compared to when only *P. irregulare* (M7) was present (Tables 12, 13). It was unexpected to find that when non-pathogenic *Pythium* species were combined with *Ph. cinnamomi* (M5), a significantly reduction in seedling survival was seen, compared to when only *Ph. cinnamomi* (M8) was present. Similarly, on lupin when native rooibos site pathogenic oomycete species (*Ph. cinnamomi, P. irregulare, P. mamillatum* and *P.* pyrilobum) were combined with only one non-pathogenic *Pythium* sp. (*Pythium* RB I) (M6) a significant reduction was seen in seedling survival, compared to when only pathogenic oomycete species were present (Table 12). On oats, co-inoculation of non-pathogenic *Pythium* species with pathogenic oomycete species (M1), resulted in a significantly higher survival rate and length of oats seedlings, compared to when only pathogenic species was present (M2) (Tables 12, 13). It should, however, be noted that the *P. mamillatum* and *Ph. cinnamomi* isolates in the pathogenic species mix were non-pathogenic towards oats (Tables 4, 5). When only one non-pathogenic *Pythium* sp. (*Pythium* RB I) was combined with the native site pathogenic oomycetes (M6) no significant suppression of disease was observed.

DISCUSSION

Several oomycete species were found associated with rooibos grown in nurseries and in a native rooibos site in the Western Cape Province of South Africa. Most of the oomycete species consisted of *Pythium* species, including *P. acanthicum, P. irregulare, P. mamillatum, P. myriotylum, P. pyrilobum*, *Pythium* RB I and *Pythium* RB II, but one *Phytophthora* species,

Ph. cinnamomi, was also identified. The rooibos nurseries and the native site had in common the fact that *P. irregulare* was the most prominent species, and that *P. mamillatum* occurred in both locations. Whether *P. acanthicum, P. myriotylum* and *Pythium* RB II, which were currently only identified in nurseries, also occur in native sites will need further investigation since the current study only included one native site. The detection of *Ph. cinnamomi* only in the native site might be due to this species being the most virulent and widespread pathogen of indigenous *Proteaceae* (Von Broembsen & Brits, 1985; Denman & Sadie, 2001), which 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 (Crous, *et al.*, 2000; McLeod *et al.*, 2009), but this is the first report of these species being associated with rooibos.

In several rooibos locations, but not all, more than one oomycete species was associated with rooibos. In the native rooibos site, five different oomycete species co-occurred including *Ph. cinnamomi, P. irregulare, P. mamillatum, P. pyrilobum* and *Pythium* RB I. Two of the rooibos nurseries contained four species, with one nursery containing *P. acanthicum, P. irregulare, P. mamillatum* and *P. myriotylum*, and the other nursery containing *P. irregulare, P. myriotylum* and *Pythium* RB I and II. In both of these nurseries *P. myriotylum* dominated the populations. The prevalence of one *Pythium* species 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 (Mazzola *et al.*, 2002; Paulitz & Adams, 2003; Suffert & Guibert, 2007; Spies *et al.*, 2011b). Biological traits such as pathogenicity, optimum temperature for mycelia growth and saprophytic survival of inoculum could explain the fluctuations in *Pythium* species composition (Suffert & Guibert, 2007). Several abiotic soil properties can also affect *Pythium* community composition in soil, including pH, calcium, magnesium and field capacity (Broders *et al*., 2009).

Pythium irregulare is an important pathogen of rooibos due to its prevalence and high virulence towards rooibos. All of the nurseries contained *P. irregulare,* for which five different PCR-RFLP groups were identified, and in several of the nurseries (58%) only *P. irregulare* was identified. *Pythium irregulare* is known as a genetically diverse group (Barr *et al.*, 1997; Matsumoto *et al.*, 2000; Lévesque & De Cock, 2004; Garzón *et al.*, 2007) and is distributed across several phylogenetic sub-clades within this species complex (Spies *et al.,*

2011a). Based on the study of Garzón *et al*. (2007), some of the rooibos *P. irregulare* isolates might be identified as *P. cryptoirregulare,* but the validity of this species has been questioned (Spies *et al.*, 2011a). The diversity in *P. irregulare* was also evident in pathogenicity studies towards oats and lupin. On oats, not all of the *P. irregulare* isolates were pathogenic, whereas on lupin some isolates differed in virulence. Spies *et al.* (2011b) also identified non-pathogenic *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.), sub-clover (*Trifolium subterraneum* L.) and wheat (Harvey *et al.*, 2001). 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) (Perneel *et al.*, 2006), tomato (*Solanum lycopersicum* L.), rye (*Secale cereal* L.), oats, wheat, sorghum (*Sorghum bicolor* [L.] Moench), peanut (*Arachis hypogaea* L.), bean (*Phaseolus vulgaris* L.), tobacco (*Nicotiana tabacum* L.), and soybean (*Glycine max* [L.] Merr) (McCarter & Littrell, 1970).

Most of the oomycete species associated with rooibos are known to have a wide host range including several vegetable and grain crops. It is thus not surprising that pathogenicity studies showed that several of the rooibos associated oomycete species were not only pathogenic toward rooibos, but also toward two nursery rotation crops (lupin and oats). Almost all oomycete species associated with rooibos, except *P. acanthicum* and the previously characterised *Pythium* RB I and RB II, was highly virulent towards rooibos causing 100% damping-off. *Pythium irregulare* is known as a highly virulent species with a very wide host range (Chamswarng & Cook, 1985; Vincelli & Lorbeer, 1990; Larkin *et al.*, 1995; Pankhurst *et al.*, 1995; Crous *et al*., 2000; Matoba *et al.*, 2008) and has been reported as a pathogen of lupin (Schultz, 1950 cited by Van der Plaats-Niterink, 1981; Sweetingham, 1989), but not of oats. *Phytophthora cinnamomi* has also been reported as a pathogen of lupin (Serrano *et al.*, 2010), and *P. myriotylum* as a pathogen of oats (McCarter & Littrell, 1970). This is thus the first report of *P. mamillatum, P. pyrilobum* and *P. myriotylum* as pathogens of lupin, and *P. irregulare* and *P. pyrilobum* as pathogens of oats. 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, cowpeas (*Vigna unguiculata* [L.] Walp.), soybean (*P. myriotylum*) and kidney bean (*Phaseolus vulgaris* L.) (*P. mamillatum* and *P. myriotylum*) (Porter, 1970; Rizvi & Yang, 1996; Matoba *et al.*, 2008). *Pythium pyrilobum* has not been reported as a pathogen of other legume crops. Although *P. irregulare* and *P. pyrilobum* have not been reported as pathogens of oats, *P. irregulare* has been reported as a

pathogen of wheat and *P. pyrilobum* as a pathogen of rice (only reduce root growth, but did not reduce shoot growth or seedling survival) (Chamswarng & Cook, 1985; Cother & Gilber, 1993). Although *P. acanthicum* has not been reported as a pathogen of oats and lupin or other legume and grain crops, it has been reported as a pathogen of tomato (Robertson, 1973 cited by Van der Plaat-Niterink, 1981) and water melon (Drechsler, 1939 cited by Van der Plaats-Niterink, 1981). 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 non-host and that can significantly reduce oomycete inoculum levels.

Non-pathogenic *Pythium* species (*P. acanthicum, Pythium* RB I and II) showed potential for suppressing damping-off caused by *P. irregulare* or a combination of oomycetes (*Ph. cinnamomi, P. irregulare, P. mamillatum, P. myriotylum* and *P. pyrilobum*) on lupin and oats, but not on rooibos. On lupin, the non-pathogenic *Pythium* species significantly reduced damping-off caused by *P. irregulare*, but not by the combination of pathogenic oomycetes or *Ph. cinnamomi*. It was unexpected that the combination of *Ph. cinnamomi* and non-pathogenic *Pythium* species resulted in a significant increase in damping-off on lupin, compared to when only *Ph. cinnamomi* was present. This suggests that there might be a synergistic interaction between *Ph. cinnamomi* and the non-pathogenic *Pythium* species, in causing disease on lupin. On oats, the non-pathogenic *Pythium* species were able to significantly reduce damping-off caused by the combination of oomycete species (*Ph. cinnamomi, P. irregulare, P. mamillatum, P. myriotylum* and *P. pyrilobum*), and when only *P. irregulare* was present. It should however be noted that in the mixture of isolates that was used, the only isolates that were pathogenic towards oats was the *P. irregulare, P. myriotylum* and *P. pyrilobum* isolates. The suppression of *Pythium* diseases by *P. acanthicum* has previously been reported, where it reduced damping-off caused by *Pythium ultimum* on cucumber (Ali-Shtayeh & Saleh, 1999) and damping-off caused by *P. irregulare* and *P. mamillatum* on sugar beet (Ribeiro & Butler, 1995). Other reports of non-pathogenic *Pythium* species that were able to reduce disease caused by pathogenic *Pythium* species include *P. periplocum* and *P. oligandrum* that reduced damping-off of sugar beet caused by *P. ultimum* (Ribeiro and Butler, 1995). Ribeiro and Butler (1995) 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 *P. acanthicum* and *P. periplocum* (Ribeiro & Butler, 1995). The three modes of parasitism implicated in disease suppression by mycoparasitic *Pythium* species include lysis,

cytoplasmic coagulation and penetration of the host at the point of contact (Laing & Deacon 1991; Ribeiro & Butler 1995).

The current study showed that *P. irregulare* is a prominent and virulent pathogen of rooibos, along with several other virulent species that were less frequently found. Most of the pathogenic rooibos species were also pathogenic towards two nursery rotation crops, lupin and oats. Of these crops, there were less species and isolates that were pathogenic towards oats, and the species were also less virulent on oats and lupin than on rooibos. The reduced number of species and isolates pathogenic towards oats compared to lupin, could explain field observations that lupin as a pre-crop significantly increased damping-off of rooibos whereas oats did not (personal communication, S. Lamprecht). The biocontrol effect of oats has previously been demonstrated in a study by Deacon and Mitchell (1985) that showed that oat roots attract and lyse zoospores of several *Pythium* species (*Pythium aphanidermatum* (Edson) Fitzp., *Pythium arrhenomanes* Drechsler, *Pythium graminicola* Subramaniam, *Pythium intermedium* de Bary, *Pythium ultimum* var. *sporangiiferum* Drechsler) and *Ph. cinnamomi*. Oat roots were also found to inhibit oospore formation and germination, due to the release of fungitoxic compounds from the roots (Deacon & Mitchell, 1985). The effective use of oats as a rotation crop will have to be evaluated further in glasshouse and field trials. The use of molecular tools to more accurately and in a high throughput manner evaluate the population shifts in oomycete species between rooibos and rotation crops will be valuable. It will also be important to determine if oats can result in an increase in non-pathogenic oomycete species that could help suppress pathogenic species on oats, and perhaps on rooibos. Ultimately this, along with disease incidence, will determine whether oats will form an important part of an integrated management strategy of rooibos damping-off caused by oomycetes. The use of non-pathogenic *Pythium* species as biocontrol agents in rooibos nurseries as a standalone treatment did not show promise, since it did not suppress damping-off on rooibos, and was also ineffective on lupin when several pathogenic species were present.

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

Location	Percentage isolates (number of isolates)							
	Ph. cinnamomi	*P. acanthicum*	*P. irregulare*[a]	*P. mamillatum*	*P. myriotylum*	*P. pyrilobum*	RB I	RB II
Nursery								
A	0	8 (1)	8 (1) PY1	0	8 (1)	0	0	0
			46 (6) PY2					
			8 (1) PY4					
			23 (3) PY5					
B	0	0	11 (1) PY2	0	0	0	0	11 (1)
			44 (4) PY4					
			33 (3) PY5					
C	0	0	5 (1) PY1	0	0	0	0	5 (1)
			28 (5) PY2					
			56 (10) PY4					
			5 (1) PY5					
D	0	0	8 (1) PY1	0	0	0	0	0
			17 (2) PY2					
			25 (3) PY4					
			17 (2) PY5					
			33 (4) PY8					
E	0	0	43 (3) PY1	0	0	0	0	0
			14 (1) PY2					
			14 (1) PY4					
			29 (2) PY5					
F	0	0	25 (1) PY4	0	0	0	0	0
			75 (3) PY5					
G	0	0	20 (1) PY1	0	0	0	0	0
			80 (4) PY2					
H	0	0	100 (5) PY4	0	0	0	0	0
I	0	17 (1)	17 (1) PY5	17 (1)	50 (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

Location	Percentage isolates (number of isolates)							
	Ph. cinnamomi	*P. acanthicum*	*P. irregulare*[a]	*P. mamillatum*	*P. myriotylum*	*P. pyrilobum*	RB I	RB II
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 14 (2) PY5	0	50 (7)	0	21 (3)	7 (1)
O	0	0	40 (2) PY 1 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 24 (27) PY2 29 (32) PY4 16 (18) PY5 4 (4) PY8	3 (3)	14 (16)	0	3 (3)	3 (3)
Native population	1 (3)	0	12 (4) PY2 33 (11) PY4	12 (4)	0	21 (7)	18 (6)	0
Total nursery + native	1 (1)	1 (2)	6 (10) PY1 20 (31) PY2 31 (48) PY4 12 (18) PY5 3 (4) PY8	4 (7)	10 (16)	4 (7)	6 (9)	2 (3)

[a] The polymerase chain reaction restriction fragment length polymorphism group (PY 1, 2, 4, 5, and 8) to which *P. irregulare* isolates belonged to are indicated.

Table 2. Analyses of variance for the effect of inoculated oomycete species (*Pythium* and *Phytophthora*), isolates and molecular groups on the mean percentage survival of three crops (lupin, oats and rooibos).

Source of variation	DF	MS	SL
Rep(Trial)	5	592.8201	<0.0001
Species	6	14050.8910	<0.0001
Isolate	32	3090.7451	<0.0001
Crop	2	283022.5733	<0.0001
Group	11	7862.3584	<0.0001
Species × Crop	12	5904.9908	<0.0001
Isolates × Crop	64	1306.7775	<0.0001
Group × Crop	22	3275.0051	<0.0001
Experimental error	489	102.1413	
Corrected total	592		

Table 3. Analyses of variance for the effect of inoculated oomycete (*Pythium* and *Phytophthora*) species, isolates and molecular groups on the mean seedling length of three crops (lupin, oats and rooibos).

Source of variation	DF	MS	SL
Rep(Trial)	5	2164.873	0.0409
Species	6	20559.048	<0.0001
Isolate	32	10454.841	<0.0001
Crop	2	1287550.793	<0.0001
Group	11	87.402311	<0.0001
Species × Crop	10	8869.774	<0.0001
Isolates × Crop	38	3829.033	<0.0001
Group × Crop	16	35.908404	<0.0001
Experimental error	344	922.603	
Sample error	3568	378.264	
Corrected total	3989		

Table 4. Percentage survival of lupin, oats and rooibos seedlings grown in soil inoculated with different *Pythium* isolates and *Phytophthora cinnamomi*, and an un-inoculated control.

		Survival (%)[b]		
Species	**Isolate[a]**	**Lupin**	**Oats**	**Rooibos**
Control	Control	74.1	94.6	94.0
Ph. cinnamomi	R8303A (PY9)	38.3	94.3	0.0
P. acanthicum	R8162U (PY10)	63.3	93.3	92.8
	R8206Q (PY10)	70.8	95.3	89.6
P. irregulare	R8282W1 (PY1)	55.0	88.0	0.0
	R8294C (PY1)	60.0	86.0	0.0
	R8162D (PY2)	52.5	82.0	0.0
	R8167A (PY2)	60.0	80.0	0.0
	R8171D (PY2)	51.6	79.0	0.0
	R8308F (PY2)	35.8	88.6	0.0
	R8129Q (PY4)	59.1	89.6	0.0
	R8304C (PY4)	65.8	89.6	0.0
	R8307N (PY4)	60.0	92.6	0.0
	R8161C (PY4)	62.5	91.3	0.0
	R8244H (PY4)	59.1	91.0	0.0
	R8168I (PY5)	58.3	84.0	0.0
	R8177A (PY5)	61.6	92.7	0.0
	R8292AE (PY5)	55.8	86.0	0.0
	R8160A (PY8)	57.5	79.0	0.0
	R8160L (PY8)	46.6	84.0	0.0
P. mamillatum	R8201C (PY6)	45.0	91.0	0.0
	R8296C (PY6)	65.0	88.0	0.0
	R8302D (PY6)	75.0	91.6	0.0
P. myriotylum	R8128T (PY3)	51.6	89.0	0.0
	R8130U (PY3)	51.6	84.3	0.0
	R8166K1 (PY3)	63.3	93.0	0.0
	R8169S (PY3)	41.6	69.6	0.0
	R8197F (PY3)	35.0	48.3	0.0
	R8204F (PY3)	48.3	57.3	0.0
P. pyrilobum	R8298A (PY7)	55.8	77.6	0.0
	R8302F (PY7)	57.5	67.6	0.0
	R8303D (PY7)	42.5	75.3	0.0
	R8303J (PY7)	26.6	66.0	0.0
LSD (*P* = 0.05)	**11.48**			

[a] The internal transcribed spacer region polymerase chain reaction restriction fragment length group (molecular group PY1 to PY10) to which each isolate belonged to are indicated in brackets.

[b] Seedling survival was evaluated two weeks after planting seeds. Values are the mean of three replicates, and data that were pooled over two trials.

Table 5. The length of lupin, oats, and rooibos seedlings grown in soil inoculated with different *Pythium* isolates and *Phytophthora cinnamomi*, and an un-inoculated control.

		Length (mm) [b]		
Species	**Isolate[a]**	**Lupin**	**Oats**	**Rooibos**
Control	Control	111.4	136.2	32
Ph. cinnamomi	R8303A (PY9)	71	151	-[c]
P. acanthicum	R8162U (PY10)	103.7	141.3	23.5
	R8206Q (PY10)	109	140.5	28.5
P. irregulare	R8282W1 (PY1)	84.9	117.6	-
	R8294C (PY1)	71	114.1	-
	R8162D (PY2)	88.6	112.6	-
	R8167A (PY2)	76.9	116.5	-
	R8171D (PY2)	62.1	115	-
	R8308F (PY2)	67.8	114	-
	R8129Q (PY4)	91.8	131.4	-
	R8304C (PY4)	88	125.5	-
	R8307N (PY4)	92.9	125.5	-
	R8161C (PY4)	97.1	129.2	-
	R8244H (PY4)	97.8	121.5	-
	R8168I (PY5)	86.4	106.5	-
	R8177A (PY5)	80.8	119.4	-
	R8292AE (PY5)	84.8	112.4	-
	R8160A (PY8)	71.9	109	-
	R8160L (PY8)	61.5	104	-
P. mamillatum	R8201C (PY6)	91.3	126	-
	R8296C (PY6)	100.5	124	-
	R8302D (PY6)	106.5	125.2	-
P. myriotylum	R8128T (PY3)	88.9	136.1	-
	R8130U (PY3)	77.6	126.1	-
	R8166K1 (PY3)	96.4	130	-
	R8169S (PY3)	68.5	124.4	-
	R8197F (PY3)	49.8	104.4	-
	R8204F (PY3)	60.2	104.8	-
P. pyrilobum	R8298A (PY7)	81	106.9	-
	R8302F (PY7)	89.1	111	-
	R8303D (PY7)	84.4	111.8	-
	R8303J (PY7)	60.2	108.6	-
LSD (*P* = 0.05)	**16.42**			

[a] The internal transcribed spacer region polymerace chain reaction restriction fragment length group (molecular group PY1 to PY10) to which each isolate belonged to are indicated in brackets.

[b] The length of lupin and oats seedlings was determined 2 weeks after sowing seeds, and after 4 weeks for rooibos. Values are the mean of three replicates, and data that were pooled over two trials.

[c] No seedlings survived.

Table 6. Percentage survival of lupin, oats, and rooibos seedlings grown in soil inoculated with different *Pythium* species, *Phytophthora cinnamomi* and an un-inoculated control.

	Survival (%)[b]		
Species[a]	**Lupin**	**Oats**	**Rooibos**
Control	74.1c	94.7a	94.0ab
Ph. cinnamomi	38.3h	94.3ab	0.0i
P. acanthicum	67.0cd	94.3ab	91.0ab
P. irregulare	56.3ef	86.4b	0.0i
P. mamillatum	61.7de	90.2ab	0.0i
P. myriotylum	48.6fg	73.6c	0.0i
P. pyrilobum	45.6gh	71.7c	0.0i
LSD	**7.90**		

[a] The values for each species is the average obtained for one to 16 isolates that were evaluated for each species. See Table 4 for the number of isolates that were evaluated for each species.

[b] Means followed by the same letter do not differ significantly at $P = 0.05$. Seedling survival was evaluated two weeks after planting seeds. Values are the mean of three replicates, and data that were pooled over two trials.

Table 7. The length of lupin, oats, and rooibos seedlings grown in soil that was inoculated with different *Pythium* species, *Phytophthora cinnamomi* and an un-inoculated control.

	Length (mm)[b]		
Species[a]	**Lupin**	**Oats**	**Rooibos**
Control	111.4de	136.2abc	32.8h
Ph. cinnamomi	71.0g	151.0a	-[c]
P. acanthicum	106.4de	140.9ab	26.0h
P. irregulare	81.9fg	117.1cde	-
P. mamillatum	100.0ef	125.1bcd	-
P. myriotylum	75.3g	120.5bcde	-
P. pyrilobum	81.1fg	109.7de	-
LSD	**22.11**		

[a] The values for each species is the average obtained for one to 16 isolates that were evaluated for each species. See Table 5 for the number of isolates that were evaluated for each species.

[b] Means followed by the same letter do not differ significantly at $P = 0.05$. The length of lupin and oats seedlings was determined 2 weeks after sowing seeds, and after 4 weeks for rooibos. Values are the mean of three replicates, and data that were pooled over two trials.

[c] No seedlings survived.

Table 8. Percentage survival of lupin, oats, and rooibos seedlings grown in soil inoculated with oomycete (*Pythium* and *Phytophthora*) species belonging to different internal transcribed spacer region polymerase chain reaction restriction fragment length (ITS PCR-RFLP) groups.

	Survival (%)[b]		
PCR-RFLP group [a]	**Lupin**	**Oats**	**Rooibos**
Control	74.2de	94.7a	94.0a
PY1 (*P. irregulare*)	57.5ghi	87.0abc	0.0l
PY2 (*P. irregulare*)	50.0ij	82.4bc	0.0l
PY3 (*P. myriotylum*)	48.6j	73.6de	0.0l
PY4 (*P. irregulare*)	61.9fg	90.9a	0.0l
PY5 (*P. irregulare*)	58.6gh	87.5abc	0.0l
PY6 (*P. mamillatum*)	61.7fg	90.2ab	0.0l
PY7 (*P. pyrilobum*)	45.6jk	71.7e	0.0l
PY8 (*P. irregulare*)	52.1hij	81.5cd	0.0l
PY9 (*Ph. cinnamomi*)	38.3k	94.3a	0.0l
PY10 (*P. acanthicum*)	67.1ef	94.3a	91.1a
LSD	**8.17**		

[a] The value for each molecular group is the average obtained for two to five isolates (see Table 4) that were evaluated for the different ITS PCR-RFLP groups. The species to which each molecular grouped belonged to is shown in brackets.

[b] Means followed by the same letter do not differ significantly at $P = 0.05$. Seedling survival was evaluated two weeks after planting seeds. Values are the mean of three replicates, and data that were pooled over two trials.

Table 9. The length of lupin, oats, and rooibos seedlings grown in soil inoculated with oomycete (*Pythium* and *Phytophthora*) isolates belonging to different internal transcribed spacer region polymerase chain reaction restriction fragment length (ITS PCR-RFLP) groups.

	Length (mm)[b]		
PCR-RFLP group [a]	**Lupin**	**Oats**	**Rooibos**
Control	111.4defg	136.2abc	32.8m
PY1 (*P. irregulare*)	78.0ijkl	115.9cdef	-[c]
PY2 (*P. irregulare*)	74.1jkl	114.6cdefg	-
PY3 (*P. myriotylum*)	75.3jkl	120.5bcde	-
PY4 (*P. irregulare*)	92.5fghij	127.9abcd	-
PY5 (*P. irregulare*)	84.0hijkl	112.8cdefg	-
PY6 (*P. mamillatum*)	100.1efghi	125.1bcd	-
PY7 (*P. pyrilobum*)	81.2ijkl	109.6defg	-
PY8 (*P. irregulare*)	67.0l	106.6defgh	-
PY9 (*Ph. cinnamomi*)	71.1kl	151.0a	-
PY10 (*P. acanthicum*)	106.4defgh	140.9ab	26.0m
LSD	**23.90**		

[a] The values for each group is the average obtained for two to five isolates (see Table 5) that were evaluated for the different internal transcribed spacer region PCR-RFLP groups. The species to which each molecular grouped belonged to is shown in brackets.

[b] Means followed by the same letter do not differ significantly at $P = 0.05$. The length of lupin and oats seedlings was determined 2 weeks after sowing seeds, and after 4 weeks for rooibos. Values are the mean of three replicates, and data that were pooled over two trials.

[c] No seedlings survived.

Table 10. Analyses of variance for the effect of co-inoculating different combinations of *Pythium* species (pathogenic and non-pathogenic) and *Phytophthora cinnamomi* on the mean percentage survival of rooibos, lupin, and oats.

Source of variation	DF	MS	SL
Block(Trials)	5	199.28519	0.0549
Species	8	8489.08333	<0.0001
Crop	2	45794.79630	<0.0001
Treatment × Crop	16	2658.04630	<0.0001
Experimental error	130	89.3006	
Corrected total	161		

Table 11. Analyses of variance for the effect of co-inoculating different combinations of *Pythium* species (pathogenic and non-pathogenic) and *Phytophthora cinnamomi* on the mean seedling length of lupin, oats and rooibos.

Source of variation	DF	MS	SL
Block(Trials)	5	3737.191	0.0095
Species	8	21143.765	<0.0001
Crop	2	627596.987	<0.0001
Treatment × Crop	16	9841.308	<0.0001
Experimental error	94	1150.777	
Sample error	954	432.311	
Corrected total	1072		

Table 12. Percentage survival of lupin, oats and rooibos seedlings grown in soil co-inoculated with different combinations of *Pythium* species (pathogenic and non-pathogenic) and *Phytophthora cinnamomi*.

	Survival (%)[a]		
Treatment	**Lupin**	**Oats**	**Rooibos**
Control	69.2ef	93.0ab	96.7a
M1 (pathogenic oomycete spp. and non-pathogenic *Pythium* spp.)	45.0hi	74.7def	0.0l
M2 (pathogenic oomycete spp.)	40.8i	53.7gh	0.0l
M3 (non-pathogenic spp.)	66.7f	90.3ab	93.0ab
M4 (*P. irregulare* and non-pathogenic *Pythium* spp.)	55.8g	79.0cde	0.0l
M5 (*Ph. cinnamomi* and non-pathogenic *Pythium* spp.)	19.2k	85.0bcd	0.0l
M6 (pathogenic oomycete spp. and *Pythium* RB I)	26.7jk	51.7gh	0.0l
M7 (*P. irregulare*)	35.8ij	88.7abc	0.0l
M8 (*Ph. cinnamomi*)	38.3i	94.3ab	0.0l
LSD	**10.80**		

[a] Means followed by the same letter do not differ significantly at $P = 0.05$. Seedling survival was evaluated two weeks after planting seeds. Values are the mean of three replicates, and data that were pooled over two trials.

Table 13. The length of lupin, oats and rooibos seedlings grown in soil co-inoculated with different combinations of *Pythium* species (pathogenic and non-pathogenic) and *Phytophthora cinnamomi*.

	Length(mm)[a]		
Treatment	**Lupin**	**Oats**	**Rooibos**
Control	120.4cd	146.5ab	33.4i
M1 (pathogenic oomycete spp. and non-pathogenic *Pythium* spp.)	90.7f	133.1bc	-[b]
M2 (pathogenic oomycete spp.)	65.0gh	101.6ef	-
M3 (non-pathogenic spp.)	125.9cd	144.1ab	33.1i
M4 (*P. irregulare* and non-pathogenic *Pythium* spp.)	92.7f	130.5c	-
M5 (*Ph. cinnamomi* and non-pathogenic *Pythium* spp.)	76.0g	157.0a	-
M6 (pathogenic oomycete spp. and *Pythium* RB I)	60.6h	112.9de	-
M7 (*P. irregulare*)	67.8gh	114.0de	-
M8 (*Ph. cinnamomi*)	71.1gh	151.0a	-
LSD	**13.45**		

[a] Means followed by the same letter do not differ significantly at $P = 0.05$. The length of lupin and oats seedlings was determined 2 weeks after sowing seeds, and after 4 weeks for rooibos. Values are the mean of three replicates, and data that were pooled over two trials.

[b] No seedlings survived.

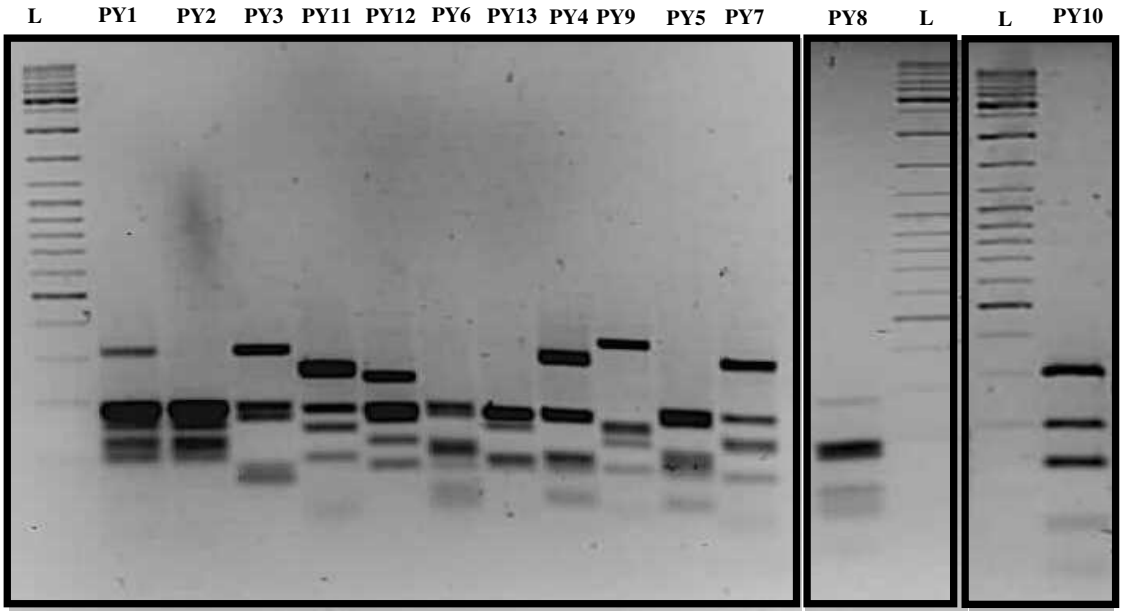

Fig. 1. Thirteen polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) groups (PY1 to PY13) that were identified among oomycete (*Pythium* and *Phytophthora*) isolates obtained from rooibos. The PCR-RFLP groups were identified by conducting a double restriction digestion with *Hinf*I and *Hha*I on PCR amplified products of the internal transcribed spacer region. A 100bp DNA ladder (L) was used to estimate the size of digested products.

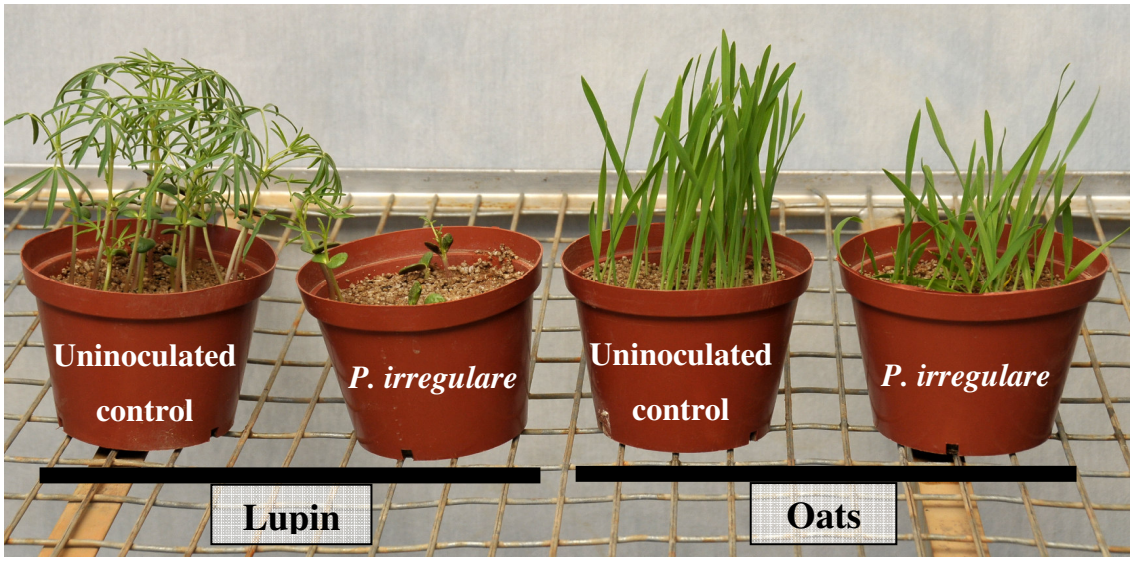

Fig. 2. Stunting and damping-off effect caused by *Pythium irregulare* (R8160L) on lupin and oats. The seedlings were planted in soil that was inoculated with *P. irregulare* and grown for two weeks.

4. SUPPRESSION OF *PYTHIUM AND PHYTOPHTHORA* DAMPING-OFF OF ROOIBOS BY COMPOST AND A COMBINATION OF COMPOST AND NON-PATHOGENIC *PYTHIUM* TAXONS

ABSTRACT

Pathogenic oomycete species including *Phytophthora cinnamomi* and several *Pythium* species (*P. irregulare, P. mamillatum, P. myriotylum,* and *P. pyrilobum*) can cause serious damping-off problems on rooibos (*Aspalathus linearis*). The management of oomycete pathogens in organic nurseries is problematic since phenylamide fungicides may not be used. In addition to pathogenic oomycetes, some non-pathogenic taxons (*P. acanthicum*, *Pythium* RB I and *Pythium* RB II) have also been identified from rooibos. Therefore, the aims of the study were to investigate whether two composts (A and B) obtained from two different producers could suppress rooibos damping-off (i) caused by *Ph. cinnamomi* and 29 *Pythium* isolates representing the known pathogenic rooibos species, (ii) when several pathogenic oomycete species were co-inoculated and (iii) when the compost treatments were combined with three non-pathogenic *Pythium* taxons (*P. acanthicum*, *Pythium* RB I and RB II). Both composts were able to suppress some, but not all of the pathogenic oomycete isolates, and differed in their ability to suppress damping-off caused by specific isolates. Compost B suppressed damping-off caused by a larger number of isolates, and it was also significantly better at suppressing damping-off of some isolates than compost A. The species that were more readily controlled by composts were *Ph. cinnamomi, P. mamillatum* and *P. pyrilobum*, whereas damping-off caused by several *P. irregulare* (> 62%) and *P. myriotylum* (> 50%) isolates was not suppressed by the composts, especially by compost A. Both composts were most effective at suppressing *Ph. cinnamomi,* although only one isolate was evaluated, since this was the only isolate where the composts improved seedling survival to a level that did not differ significantly from the un-inoculated control. Neither of the composts as a standalone treatment could suppress damping-off when several pathogenic oomycete species (*Ph. cinnamomi, P. irregulare, P. mamillatum, P. myriotylum* and *P. pyrilobum*) were co-inoculated. However, when non-pathogenic *Pythium* taxons (*P. acanthicum*, *Pythium* RB I and RB II) were combined with either of the two composts, damping-off caused by the aforementioned combination of pathogenic species was significantly suppressed. Similarly, damping-off caused by a *P. irregulare* isolate that was not suppressed by either of the

composts alone, was significantly suppressed when the two composts were inoculated with the non-pathogenic *Pythium* taxons.

INTRODUCTION

Rooibos (*Aspalathus linearis* (N.L. Burm.) R. Dahlgr.), an important crop in South Africa from which herbal tea is made, is very susceptible to soilborne oomycete pathogens including *Pythium* and *Phytophthora*. These pathogens have the potential to cause damping-off of rooibos seedlings in nurseries, resulting in considerable stand losses. Several oomycete species are involved, with *P. irregulare* Buisman being the most widespread in nurseries. *Pythium irregulare* is highly virulent towards rooibos, causing 100% damping-off in artificial inoculation studies. Other species that are also highly virulent, but less widespread, include *Phytophthora cinnamomi* Rands and the *Pythium* species *P. mamillatum* Meurs, *P. myriotylum* Drechsler and *P. pyrilobum* Vaartaja. Although *Ph. cinnamomi* and *P. pyrilobum* have thus far only been identified from native rooibos, these pathogens might also be present in nurseries that have not yet been surveyed (Chapter 3). In rooibos nurseries, the management of oomycete induced damping-off should thus target all these diverse species.

The management of soilborne oomycetes is difficult, and only a few management strategies are available. The use of fungicides, such as the phenylamide fungicides metalaxyl and mefenoxam, provides excellent control of several soilborne oomycete pathogens (Erwin & Ribeiro, 1996). In rooibos nurseries, these fungicides applied as a seed treatment may have potential for suppressing damping-off, since mefenoxam is registered as a seed treatment for several small seeded crops. However, resistance to phenylamide fungicides is well known (Bruin & Edgington, 1981; Mazzola *et al.*, 2002), and it should thus form part of an integrated strategy. Another problem with the use of fungicides is that it is not allowed in organic production. Some countries prefer to import organically produced rooibos tea, because of their health-conscious consumers (Personal communication, S. C. Lamprecht, ARC-Plant Protection Research Institute, South Africa). Other management approaches that are available include water management (Erwin & Ribeiro, 1996), organic amendments such as compost and green manures (De Ceuster & Hoitink, 1999), rotation crops (Fry, 1982; Havlin *et al.*, 1990), physical methods such as soil solarization (Katan, 2000) and biological control (Naseby *et al.*, 2000; Quagliotto *et al.*, 2009). The rotation crops (lupin [*Lupinus*

angustifolius L.] and oats [*Avena sativa* L.]) that have traditionally been used, and are still being used, in rooibos nurseries are hosts to most of the rooibos oomycete pathogens, but they are less susceptible than rooibos (Chapter 3). Preliminary field trials have shown that oats as a rotation crop causes less damping-off of rooibos seedlings than when lupin is used, and thus shows promise for use in an integrated management strategy (personal communication, S.C. Lamprecht). Oats as a rotation crop could help to cause a decline in inoculum, but it cannot suffice as a standalone management practice, since *Pythium* species have a relative high saprophytic survival capacity, wide host range and forms efficient survival structures (Van der Plaats-Niterink, 1981), making them less amendable to management through rotation (Fry, 1982). The evaluation and implementation of additional management strategies are thus required.

The use of compost as part of an integrated management approach in rooibos nurseries holds promise, since it not only has the potential to suppress disease, but also has other benefits for crop production. Compost serves as a nutrient source for plants and as such can contribute to the recycling of waste and reduce the use of non-renewable artificial fertilizers (Termorshuizen *et al*., 2006). Another benefit is the improvement of the physical structure of soils that contributes towards improved soil health (Idowu *et al.*, 2008). Organic amendments, such as compost, also enhance soil microbial biomass and activity, since it is rich in labile carbon fractions that serve as an energy source for microorganisms. It is this enhanced general microbial activity, which often includes microorganisms antagonistic toward plant pathogens, which contributes to disease suppression (Janvier *et al*., 2006). The suppression of disease by compost can also be the result of induced systemic resistance in plants, although very few composts (<10%) induce systemic resistance in plants (Hoitink & Boehm, 1999).

The use of compost to suppress soilborne plant pathogens has been widely studied and is well known (De Ceuster & Hoitink, 1999; Ben-Yephet & Nelson, 1999; Aryantha *et al.*, 2000; Hoitink & Changa, 2004; Scheuerell *et al.*, 2005; Termorshuizen *et al.*, 2006). Disease suppression is unfortunately not always consistent and can be influenced by various factors. One of these is the composition of composts, since compost can be made from different kinds of waste materials and raw feedstocks that vary in chemical, physical and biotic composition (Termorshuizen *et al.*, 2006). Other factors influencing disease suppression include the rate of application, the degree of maturity and the specific pathogens

involved (Janvier *et al*., 2006). The latter is important, for example most composts naturally suppress *Pythium* and *Phytophthora* root rots, whereas only 20% of compost naturally suppress *Rhizoctonia* damping-off (Hoitink & Boehm, 1999).

Biological control, defined here as the use of microorganisms to suppress plant pathogens, of Pythium damping-off diseases has been studied widely and can be effective (Hadar *et al.*, 1984; Loper, 1988; Whips & Lumsden, 1991; Naseby *et al.*, 2000; Nelson *et al.*, 2004; Quagliotto *et al.*, 2009). It should thus also be considered for inclusion in an integrated management strategy in rooibos nurseries. Microorganisms that have shown some potential for suppressing *Pythium* include *Enterobacter cloacae* (Jordan) Hormaeche and Edwards, *Gliocladium virens* Miller *et al., Pseudomonas fluorescens* (Trevisan) Migula, *Pythium acanthicum* Drechsler, *P. oligandrum* Drechsler, *P. periplocum* Drechsler, *Streptomyces griseoviridis* Anderson *et al., Trichoderma koningii* Oud, and *T. harzianum* Rifai on various crops (Hadar *et al.,* 1984; Whipps & Lumsden, 1991; Ribeiro & Butler, 1995; Ali-Shtayeh & Saleh, 1999; Quagliotto *et al.,* 2009). The ecological processes that determine the efficacy of biological control agents are complex, and have resulted in variable control efficacies and the limited use of biocontrol in field crops regardless of substantial research efforts (Xu *et al.*, 2011). Therefore, biocontrol agents have also been used in combination with fungicides or cultural practices in an integrated management strategy (Xu *et al.*, 2011). The integrated use of biocontrol agents with compost was first developed by Hoitink and Boehm (1999) for plant growing media. This integrated management strategy has not been studied widely and have yielded variable results (Nakasaki *et al*., 1998; Coventry *et al.*, 2006; Noble, 2011; Pugliese *et al*., 2011).

The overall aim of the present study was to evaluate the potential of composts, sourced from two different producers, and composts combined with non-pathogenic *Pythium* taxons, to suppress oomycete induced damping-off of rooibos. Due to the diversity in pathogenic oomycete species on rooibos, it was important to determine whether the composts could suppress all the different species and isolates within species. Therefore, 30 isolates representing all of the known pathogenic species (*Ph. cinnamomi, P. irregulare, P. mamillatum, P. myriotylum* and *P. pyrilobum*) were evaluated. The other aim of the study was to determine whether a combination of compost and non-pathogenic *Pythium* taxons (*P. acanthicum, Pythium* RB I and *Pythium* RB II) was able to suppress damping-off caused by i) a *P. irregulare* isolate that was not suppressed by the composts alone, (ii) *Ph. cinnamomi* and

(iii) a combination of several pathogenic oomycete species (*Ph. cinnamomi, P. irregulare, P. mamillatum, P. myriotylum* and *P. pyrilobum*). Lastly, the ability of composts and a non-pathogenic *Pythium* taxon, *Pythium* RB I, which was identified from a native rooibos site, to suppress damping-off caused by pathogenic oomycete spp. (*Ph. cinnamomi, P. irregulare, P. pyrilobum, P. mamillatum*) identified from a native rooibos site, was also evaluated.

MATERIALS AND METHODS

***Pythium* and *Phytophthora* isolates.** A total of 33 oomycete isolates, including one *Phytophthora* isolate and 32 *Pythium* isolates were used in the study. The isolates were obtained from two previous studies that characterized the pathogenicity of the isolates towards rooibos, lupin and oats (Chapter 2, 3). The isolates included the following pathogenic species: *Ph. cinnamomi* (1 isolate), *P. irregulare* (16 isolates), *P. mamillatum* (3 isolates), *P. myriotylum* (6 isolates) and *P. pyrilobum* (4 isolates) (Table 2). The non-pathogenic species included *P. acanthicum* (R8206Q), *Pythium* RB I (STE-U 7555) and *Pythium* RB II (STE-U 7548). All isolates were stored as CMA culture plugs in sterile de-ionised water containing grass blades, V8-agar (Galindo & Gallegly, 1960) plugs in sterile de-ionised water, and potato-carrot agar (Dhingra & Sinclair, 1985) slant cultures**.**

Effect of two different composts on the emergence of rooibos seedlings when inoculated with pathogenic oomycete isolates. Sand-bran inoculum was prepared for each of the 30 pathogenic isolates as previously described (Lamprecht, 1986; Chapter 2). The inoculum was used to introduce each isolate at a concentration rate of 0.05% (wet wt/wet wt), into (i) pasteurised planting medium (sand:soil:perlite [1:1:1]), (iii) pasteurized planting medium amended with 25% v/v of compost A and (iii) pasteurized planting medium amended with 25% v/v of compost B. The controls consisted of composts (A or B) amended (25% v/v) and un-amended planting media that was inoculated with 0.05% sand/bran, which was inoculated with un-colonized agar plugs. Plastic pots (13 cm diameter) were filled with approximately 800g of the inoculated planting media. Sterile doweling rods (1 cm diameter) were used to make 10 holes in each pot to a depth of 1.5 cm. Five rooibos seeds were planted in each hole giving a total of 50 seeds per pot. Each treatment contained three pots (replicates). The trials were conducted in a glasshouse at approximately 18 °C night and 27 °C day temperatures. The experiment was a complete randomized block design. The entire

trial was conducted twice, with different batches of compost A and B being used in the repeat trials. The trial was evaluated after two weeks by determining the percentage seedling survival. Re-isolations were made from the seedlings to fulfill Koch's postulates (Tewoldemedhin *et al.*, 2011).

Effect of two different composts on the emergence of rooibos seedlings when co-inoculated with non-pathogenic and pathogenic oomycetes. The assays were conducted in a similar manner than described in the section above, except that different isolates were co-inoculated (each isolate at a rate of 0.05% [wet wt/wet wt]) into the un-amended or composts amended pasteurized planting media. The isolates that were used in the co-inoculation studies included *Ph. cinnamomi* R8303A, *P. acanthicum* R8206Q, *P. irregulare* R8308F, *P. mamillatum* R8201C, *P. myriotylum* R8197F, *P. pyrilobum* R8198A, *Pythium* RB I STE-U 7555 and *Pythium* RB II STE-U 7548. The eight treatments that were included in the trial consisted of: M1, pathogenic oomycete spp. (*Ph. cinnamomi, P. irregulare*, *P. mamillatum*, *P. myriotylum* and *P. pyrilobum*) and non-pathogenic *Pythium* taxons (*P. acanthicum*, *Pythium* RB I and RB II); M2, pathogenic oomycete spp. (*Ph. cinnamomi, P. irregulare*, *P. mamillatum*, *P. myriotylum* and *P. pyrilobum*); M3, non-pathogenic *Pythium* taxons (*P. acanthicum*, *Pythium* RB I and RB II), M4, *P. irregulare* and non-pathogenic *Pythium* taxons (*P. acanthicum*, *Pythium* RB I and RB II); M5, *Ph. cinnamomi* and non-pathogenic *Pythium* taxons (*P. acanthicum*, *Pythium* RB I, and RB II; M6, oomycete spp. from a native site that included four pathogenic spp. (*Ph. cinnamomi, P. irregulare, P. mamillatum* and *P. pyrilobum*) and one non-pathogenic taxon (*Pythium* RB I); M7, *P. irregulare*; and M8, *Ph. cinnamomi*.

Statistical analyses. The statistical analyses of data were performed for both trials. Levene's test was conducted for homogeneity of trials to test for trial variances between repeats (Levene, 1960). The data were subjected to analysis of variance using SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC, USA). The Shapiro-Wilk test was done to test for normality (Shapiro & Wilk, 1965) and Fisher's least significant difference (LSD) for percentage survival (Ott, 1998) was also calculated to compare means at the 5% significance level.

RESULTS

Effect of two different composts on the emergence of rooibos seedlings when inoculated with pathogenic oomycete isolates. Variance for the data from the two repeat trials was comparable and therefore data from the two trials were combined. Analyses of variance were conducted on the mean percentage seedling survival of rooibos. Significant interactions were recorded between species and compost ($P < 0.0001$) and isolate and compost ($P < 0.0001$) (Table 1).

The assays showed that composts A and B were able to suppress damping-off caused by pathogenic oomycete isolates, but that isolates within species varied in their response to composts (Table 2). For most of the *P. irregulare* isolates, with exception of a few, damping-off was not significantly suppressed by compost A (87% of isolates not significantly suppressed) or B (62% of isolates not significantly suppressed), with compost B being more effective in reducing damping-off. Compost A only significantly reduced damping-off caused by *P. irregulare* isolates R8292AE and R8244H, whereas compost B significantly suppressed these isolates and four other *P. irregulare* isolates (R8167A, R8177A, R8294C and R8304C). Composts A and B were both able to suppress damping-off caused by two (R8128T and R8166K1) of the six *P. myriotylum* isolates. However, compost B was significantly better at reducing the percentage damping-off caused by the two *P. myriotylum* isolates, in addition to also significantly suppressing damping-off caused by *P. myriotylum* isolate R8130U. Almost all of the *P. pyrilobum* isolates were significantly suppressed by both composts, with the exception of isolate R8302F, which was not suppressed by compost A. Compost B was significantly better at suppressing damping-off caused by *P. pyrilobum* isolate R8298A, compared to compost A. Both composts were able to significantly reduce damping-off caused by *Ph. cinnamomi* and all three *P. mamillatum* isolates, with compost B being significantly better at suppressing damping-off caused by two (R8201C and R8302D) of the three *P. mamillatum* isolates compared to compost A (Table 2). When the data of isolates were pooled per species, the ability of (i) compost B to cause significantly better suppression of damping-off caused by some pathogenic oomycete species (*P. myriotylum* and *P. pyrilobum*) than compost A was evident and (ii) *P. irregulare* only being suppressed significantly by compost B (Table 3).

Effect of two different composts on the emergence of rooibos seedlings when co-inoculated with non-pathogenic and pathogenic oomycetes. Based on Levene's variance ratio test, variance for the data from the two repeat trials was comparable and the data of the two trials could be combined. Analyses of variance was conducted on the mean percentage survival and showed a significant interaction between treatment and compost ($P < 0.0001$) (Table 4).

Non-pathogenic *Pythium* taxons in combination with composts significantly improved the suppression of damping-off (Table 5). Composts A and B could not significantly suppress damping-off when several pathogenic oomycete species (*Ph. cinnamomi, P. irregulare, P. mamillatum, P. myriotylum* and *P. pyrilobum*) were co-inoculated (treatment M2). However, when the three non-pathogenic taxons (*P. acanthicum, Pythium* RB I and RB II) were co-inoculated into the composts, significant suppression of damping-off caused by the combination of pathogenic species was obtained (treatment M1). Similarly, the *P. irregulare* isolate that was not suppressed by either of the composts alone (treatment M7), was significantly suppressed when the three non-pathogenic taxons were co-inoculated into composts A and B (treatment M4). Both composts were very effective in suppressing *Ph. cinnamomi* (treatment M8), and survival of seedlings were improved to levels that did not differ significantly from the un-inoculated control. Therefore, the addition of non-pathogenic *Pythium* taxons (treatment M5) did not improve disease control of *Ph. cinnamomi* further. In all the treatments that included non-pathogenic taxons, compost B was only superior to compost A in suppressing damping-off in the treatment where one non-pathogenic taxon (*Pythium* RB I) was co-inoculated with four pathogenic oomycete species from a native rooibos site (*Ph. cinnamomi, P. irregulare, P. mamillatum* and *P. pyrilobum*) (treatment M6).

DISCUSSION

The present study demonstrated that two composts (A and B) from different sources can suppress damping-off of rooibos caused by *Pythium* and *Phytophthora.* Moreover, when non-pathogenic *Pythium* species were combined with the compost treatments containing several pathogenic *Pythium* species (*P. irregulare, P. mamillatum, P. myriotylum,* and *P. pyrilobum*) or *P. irregulare*, the survival of seedlings were significantly increased (~ 50%). In

contrast, compost as a standalone treatment could not suppress damping-off when several pathogenic *Pythium* species were co-inoculated. Another important finding was that when isolates were inoculated individually, isolates within pathogenic species varied in their response to being suppressed by both composts. Most notably was that the majority of the *P. irregulare* isolates was not suppressed by either of the composts as well as several *P. myriotylum* isolates. In contrast, compost B was able to suppress all *P. mamillatum* and *P. pyrilobum* isolates, and both composts were able to suppress *Ph. cinnamomi* to levels where seedling survival did not differ significantly from the uninoculated control. The two composts differed in their ability to suppress damping-off, with compost B not only suppressing more isolates, but also causing a significantly better suppression of damping-off than compost A for several isolates.

Variation in disease suppression by composts among isolates within the same *Pythium* species has not been reported widely. This may impart be due to many studies only evaluating a few isolates for a specific species (Erhart *et al.*, 1999; Scheuerell *et al.*, 2005). In the current study, the highest variation in suppression of isolates by compost was identified among *P. irregulare* (16 isolates) isolates, where only 13% of the isolates were suppressed by compost A and 38% by compost B. Variation was also observed among *P. myriotylum* (6 isolates) isolates, with 33% (compost A) to 50% (compost B) of the isolates being suppressed by the composts. This could be due to more isolates being evaluated for the two aforementioned species, compared to *P. mamillatum* (3 isolates) where all isolates were suppressed by both composts, and *P. pyrilobum* (4 isolates) where compost B suppressed all the isolates and compost A suppressed three of the isolates. Since only one isolate of *Ph. cinnamomi* was evaluated, the within species variation in suppression by compost is unknown for this species on rooibos. It was, however, notable that both composts were most effective at suppressing *Ph. cinnamomi*. In contrast to the current study, Ben-Yephet and Nelson (1999) did not find variation in suppression among isolates of *P. irregulare* (9 isolates) and *P. myriotylum* (7 isolates) by leaf compost. They did, however, find variation in suppression among isolates of *P. aphanidermatum* (11 isolates). This, unfortunately complicates the use of composts for control of Pythium damping-off.

Although the suppression of plant diseases by compost is well known, variation in the effectiveness of batches of composts has been reported widely (McKellar and Nelson, 2003; Chen & Nelson, 2008; Termorshuizen *et al.,* 2006; Noble, 2011). In the current study, the

two composts (A and B) differed in their efficacy in suppressing damping-off, with compost B being superior in suppressing Pythium damping-off of rooibos. This superior disease suppression of compost B was seen when individual pathogenic oomycete isolates was inoculated. Several factors can contribute to variation in disease suppression by composts, such as the type of feedstocks and waste materials used (Craft & Nelson, 1996; Ben-Yephet & Nelson, 1999; Termorshuizen *et al.*, 2006), and variation in the microbial community composition (Chen *et al.,* 1988a, b; Mandelbaum *et al.,* 1988). Unfortunately, information on the feedstocks that were used for making compost A and B in the current study was not made available. The factor that has been studied most, and is also considered to be one of the most important in disease suppression, is the contribution of microbial communities (Noble & Coventry, 2005; Chen & Nelson, 2008; Noble, 2011). Our current lack of understanding of the specific microbial communities that contribute to disease suppression by compost is hampering efforts to develop compost that consistently suppresses disease. In agricultural soils, the contribution of indigenous soil microbial communities that respond to compost amendments is another complicating factor (McKellar & Nelson, 2003). With damping-off diseases one important prerequisite is that the microbial community must suppress *Pythium* within the first few hours of seed germination, since Pythium damping-off pathogens respond rapidly (germinate and infect) to seed exudates released from germinating seeds (McKellar & Nelson, 2003). The mechanisms of disease suppression by composts for damping-off diseases can include a microbial community that rapidly responds to exudates from germinating seeds that (i) destroy signals from seed exudates that stimulate germination of the pathogens, as has been found for *Pythium ultimum* Trow (McKellar & Nelson, 2003), (ii) act antagonistically towards germinated pathogen propagules through competition, antibiosis, hyperparasitism and/or (iii) induce systemic acquired resistance in the host plant when seedlings get older (Hoitink & Boehm, 1999).

The suppression of oomycete damping-off of rooibos was significantly improved when compost treatments were combined with non-pathogenic *Pythium* taxons (*P. acanthicum, Pythium* RB I and RB II). A previous study (Chapter 3), along with the current study, showed that non-pathogenic *Pythium* taxons alone cannot suppress a combination of pathogenic oomycete species (*Ph. cinnamomi, P. irregulare, P. mamillatum, P. myriotylum* and *P. pyrilobum*), *Ph. cinnamomi* or *P. irregulare*. Similarly, the composts as standalone treatments cannot suppress damping-off in the presence of several pathogenic oomycete species, and only some *P. irregulare* isolates were suppressed. However, when non-

pathogenic *Pythium* taxons were added to composts, significant suppression of damping-off was achieved where several pathogenic oomycete species were co-inoculated, and where a *P. irregulare* isolate that was not be suppressed by composts alone was inoculated. This is the first report of the combination of non-pathogenic *Pythium* taxons and compost being able to suppress a seedling damping-off disease. The improvement of disease suppression by inoculating compost with other biocontrol agents have, however, been reported for a few other pathogens (*Rhizoctonia solani* Kühn and *Sclerotium cepivorum* Berk.) on beans (*Phaseolus vulgaris* L.), onion (*Allium cepa* L.) and turf grass (Nakasaki *et al*., 1998; Convenry *et al*., 2006; Pugliese *et al*., 2011). In contrast, Pugliese *et al.* (2011) found that the co-inoculation of biocontrol agents, such as *T. harzianum,* into compost did not influence the suppression of *P. ultimum* and *Phytophthora nicotianae* Breda de Haan. Similarly, the combination of non-pathogenic *Fusarium* species and compost could not increase suppression of *F. oxysporum* f.sp. *basilici* (Dzidzariya) Armstr. & Armstr. on basil (*Octimum basilicum* L.) (Pugliese *et al.*, 2011). In the current study it was notable that the superior nature of compost B to suppress damping-off when compared to compost A, was no longer evident when the composts were inoculated with non-pathogenic *Pythium* species. This may suggest that the combination of biological control agents with compost can improve the consistency in disease suppression by compost, as suggested by Noble (2011). The mechanisms involved in the improvement of disease suppression of compost by non-pathogenic *Pythium* taxons could be due to nutrients in the composts enhancing the saprophytic ability of the non-pathogenic *Pythium* taxons with subsequent increases in their population size, which could lead to their dominance in the infection court (spermosphere) early in the germination process of seeds. Nutrient competition during seed germination, when sugars and amino acids leak out of the seeds (Hoitink & Changa, 2004), is important for suppressing Pythium damping-off pathogens, since sporangia of several *Pythium* species germinate rapidly in response to seed exudates, followed by immediate infection (Nelson, 1990; Paulitz, 1991; Kageyama & Nelson, 2003; Nelson *et al.*, 2004).

The use of a large volume of compost most likely contributed to the success in suppression of damping-off in some treatments in the current study, where composts were applied at a rate of 25% v/v. Noble (2011) in a literature study found that in container experiments where soil was amended with ≥ 20% v/v compost, 75% (59/79) of the experiments resulted in disease suppression. Similarly, a large quantity (≥ 15 t/ha) of compost

added to soil in field studies, resulted in a high (76%) success rate in disease suppression (Noble, 2011).

The current study showed that composts can be effective in controlling damping-off caused by a combination of pathogenic oomycete species and by *P. irregulare,* when combined with non-pathogenic *Pythium* taxons. The fact that compost as a standalone treatment could not suppress several pathogenic oomycete isolates, notably isolates of *P. irregulare,* could contribute to variation in disease suppression across different sites if only compost is applied as a management strategy. Future research should elucidate the mechanisms involved in disease suppression caused by a combination of pathogenic and non-pathogenic oomycete species. It will also be important to determine whether the non-pathogenic *Pythium* taxons combined with compost will provide consistent damping-off suppression under field conditions across different soil types and climatic conditions. It will furthermore be important to determine why only some isolates within certain *Pythium* species are suppressed by compost.

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Table 1. Analyses of variance for the effect of two composts (A and B) on mean percentage survival of rooibos seedlings in planting media amended and un-amended with composts, and that were inoculated with different pathogenic oomycete species (*Pythium* and *Phytophthora*).

Source of variation	DF	MS	SL
Block(Trial)	5	952.6824	0.0173
Species	5	31429.8067	<0.0001
Isolate	30	7380.7914	<0.0001
Compost	2	43075.1470	<0.0001
Species × Compost	10	3260.0067	<0.0001
Isolate × Compost	60	1386.7692	<0.0001
Experimental error	460	183.1578	
Corrected total	557		

Table 2. Percentage survival of rooibos seedlings that were planted in composts (A and B) amended and un-amended planting media that were inoculated with different *Pythium* isolates and *Phytophthora cinnamomi*.

		Survival (%)[a]		
Species	**Isolate**	**Control**	**Compost A**	**Compost B**
Control	Control	94.0a	89.0ab	95.7a
Ph. cinnamomi	R8303A	0.0t	76.7bcd	83.3abc
P. irregulare	R8129Q	0.0t	6.0st	8.7rst
	R8160A	0.0t	0.0t	13.7pqrst
	R8160L	0.0t	0.0t	4.0st
	R8161C	0.0t	13.3p-t	7.7rst
	R8162D	0.0t	5.3st	11.7q-t
	R8167A	0.0t	0.0t	17.0o-s
	R8168I	0.0t	3.0st	8.3rst
	R8171D	0.0t	3.0st	3.7st
	R8177A	0.0t	6.0st	54.0g-k
	R8282W1	0.0t	0.0t	8.7rst
	R8292AE	0.0t	50.0g-k	53.7g-k
	R8294C	0.0t	6.7rst	26.3m-q
	R8307N	0.0t	5.3st	7.7rst
	R8308F	0.0t	3.3st	4.7st
	R8244H	0.0t	32.7lmn	57.0f-i
	R8304C	0.0t	6.7rst	16.3p-s
P. mamillatum	R8201C	0.0t	22.0n-r	40.7j-m
	R8296C	0.0t	39.3klm	32.0lmno
	R8302D	0.0t	45.3i-l	65.3d-g
P. myriotylum	R8128T	0.0t	52.7g-k	73.0cde
	R8130U	0.0t	5.0st	55.3f-j
	R8166K1	0.0t	27.3m-p	70.1c-f
	R8169S	0.0t	7.0rst	8.7rst
	R8197F	0.0t	0.0t	0.0t
	R8204F	0.0t	0.0t	11.0q-t
P. pyrilobum	R8298A	0.0t	25.7m-q	63.3d-g
	R8302F	0.0t	14.7p-t	21.7n-r
	R8303D	0.0t	62.7d-g	61.0e-h
	R8303J	0.0t	45.0i-l	47.0h-l
LSD	**15.61**			

[a] Means in columns and rows followed by the same letter do not differ significantly at $P = 0.05$. The plant medium was amended with two different composts (A and B) at a rate of 25% v/v. Seedling survival was evaluated two weeks after planting rooibos seeds. Values are the mean of three replicates, and data that were pooled over two trials.

Table 3. Percentage survival of rooibos seedlings in composts (A and B) amended and un-amended planting media that were inoculated with different *Pythium* species and *Phytophthora cinnamomi*.

	Survival (%)[b]		
Species[a]	**Control**	**Compost A**	**Compost B**
Control	94.0ab	89.0ab	95.7a
Ph. cinnamomi	0.0g	76.7c	83.3bc
P. irregulare	0.0g	8.8fg	18.9f
P. mamillatum	0.0g	35.5e	46.0de
P. myriotylum	0.0g	15.3f	36.4e
P. pyrilobum	0.0g	37.0e	48.2d
LSD	**10.70**		

[a] The values for each species is the average obtained for one to 16 isolates that were evaluated for each species. See Table 2 for the number of isolates that were evaluated for each species.

[b] Means in columns and rows followed by the same letter do not differ significantly at $P = 0.05$. The planting media were amended with two different composts (A and B) at a rate of 25% v/v. Seedling survival was evaluated two weeks after planting rooibos seeds. Values are the mean of three replicates, and data that were pooled over two trials.

Table 4. Analyses of variance for the effect two composts (A and B) on mean percentage survival of rooibos seedlings that were co-inoculated with pathogenic oomycete species (*Pythium* and *Phytophthora*) and non-pathogenic *Pythium* taxons.

Source of variation	DF	MS	SL
Block(Trial)	5	1474.4691	<0.0001
Treatment	8	19601.0802	<0.0001
Compost	2	17352.3210	<0.0001
Treatment × Compost	16	2719.5710	<0.0001
Experimental error	130	262.1307	
Corrected total	161		

Table 5. Percentage survival of rooibos seedlings that were planted in composts (A and B) amended and un-amended planting media that were inoculated with different combinations of pathogenic and non-pathogenic oomycete taxons (*Pythium* and *Phytophthora*).

	Survival (%)[a]		
Species	**Control**	**Compost A**	**Compost B**
Control	96.7a	89.7ab	79.3ab
M1 (five pathogenic oomycete spp. and three non-pathogenic *Pythium* taxons)	0.0d	40.0c	49.0c
M2 (five pathogenic oomycete spp.)	0.0d	0.3d	9.3d
M3 (three non-pathogenic *Pythium* taxons)	93.0ab	92.7ab	89.3ab
M4 (*P. irregulare* and three non-pathogenic *Pythium* taxons)	0.0d	39.3c	55.3c
M5 (*Ph. cinnamomi* and three non-pathogenic *Pythium* taxons)	0.0d	83.7ab	75.3b
M6 (non-pathogenic *Pythium* RB I and four pathogenic oomycete spp. from native rooibos site)	0.0d	9.7d	48.0c
M7 (*P. irregulare*)	0.0d	3.3d	4.7d
M8 (*Ph. cinnamomi*)	0.0d	76.7b	83.3ab
LSD	**18.50**		

[a] Means in columns and rows followed by the same letter do not differ significantly at $P = 0.05$. The planting media were amended with composts (A and B) at a rate of 25% v/v. Seedling survival was evaluated two weeks after planting rooibos seeds. Values are the mean of three replicates, and data that were pooled over two trials.

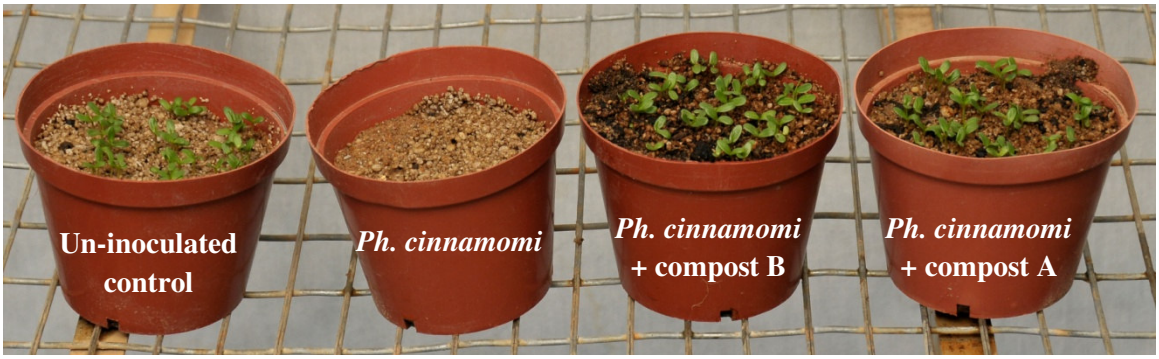

Fig. 1. Effect of two composts (A and B) on damping-off caused by *Phytophthora cinnamomi* (R8303A) on rooibos seedlings. The un-inoculated control did not receive any compost or *Phytophthora* inoculum.

Fig. 2. Effect of two composts (A and B) on damping-off caused by *Pythium irregulare* (R8292AE) on rooibos seedlings. The un-inoculated control did not receive any compost or *Pythium* inoculum.