Risk assessment of the *Acacia cyclops* dieback pathogen, *Pseudolagarobasidium acaciicola*, as a mycoherbicide in the South African strandveld and limestone fynbos

by

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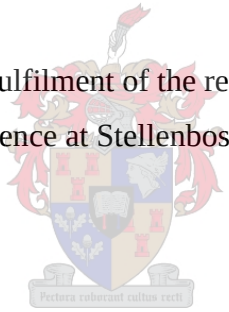

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Summary

Acacia cyclops, an invasive weed in South Africa, was initially imported to stabilize the sand dunes in the southern Cape. The spread of *A. cyclops* is a major threat to the fragile biodiversity of the strandveld and limestone fynbos vegetation. *Acacia cyclops* dieback has been observed for some time, although the causative agent, *Pseudolagarobasidium acaciicola*, has only recently been described. This fungus is nominated for development as a mycoherbicide to control *A. cyclops*. Although current biological and mechanical control efforts are proving to be partially effective, *A. cyclops* is still causing major damage to natural ecosystems. The introduction of a mycoherbicide would increase the cost effectiveness of controlling this weed in the long term. The majority of the literature that was reviewed supports the use of mycoherbicides as biocontrol agents, especially when taking into account the decrease in acceptance and availability of chemical control agents. Considering that the *Pseudolagarobasidium* genus consists of saprobes, opportunistic facultative pathogens and endophytes, *P. acaciicola* is predicted to have similar biological characteristics. The species is also highly likely to be indigenous, although with a wider distribution range than previously envisaged. Strict precautions should still however be taken to ensure that non-target species will not be threatened. This study consists of a unique risk assessment comprising different sections. A field survey was performed to record disease incidence among indigenous woody plant species around 100 diseased *A. cyclops* trees. Subsequently, DNA extractions were made from the roots of the diseased indigenous plants and *A. cyclops* trees to verify the presence of *P. acaciicola*. Of the 2432 indigenous woody plants observed, 22 (0.9%) were dead or dying, while *P. acaciicola* was detected in 10 of these (0.4%), representing six species. *Pseudolagarobasidium acaciicola* was detected in 47% of the *A. cyclops* trees. Although *P. acaciicola* could be a weak pathogen in a broad range of indigenous plant species, the extremely low disease incidence is an indication of a low level of risk associated with using *P. acaciicola* as a mycoherbicide. Additionally, pathogenicity trials on indigenous

plant species were conducted to give an indication of host susceptibility. A total of 30 indigenous plant species were wound inoculated at two field sites, and potted plants representing 17 indigenous plant species were wound and soil inoculated in a nursery. The optimum growth temperature for *P. acaciicola* was determined in order to understand it's seasonal and landscape preference. Mortality was recorded in five of nine indigenous Fabaceae species, while a single plant each of four other non-Fabaceae species died after inoculation. No plants outside the Fabaceae family died in the field. Only *A. cyclops* seedlings died following soil inoculation. Longitudinal sections of stem inoculated plants revealed no systemic infection in Fabaceae species that survived inoculation. Infection in susceptible Fabaceae species was generally more extensive than infection in susceptible non-Fabaceae species. The optimum growth rate for *P. acaciicola* was determined at 35°C, indicating an adaptation to summer conditions. Indigenous Fabaceae species do display greater susceptibility than species from other families, indicating some level of specificity, although susceptible species can not be phylogenetically circumscribed. Aside from being a facultative pathogen on *A. cyclops*, results from this study suggest that *P. acaciicola* is primarily a saprophyte and an occasional opportunistic pathogen on some indigenous Fabaceae, possibly only being a weak opportunistic pathogen on some non-Fabaceae species. However, the risk of not effectively managing *A. cyclops* populations in these threatened vegetation types outweighs the risk associated with using *P. acaciicola* as a mycoherbicide. Therefore the use of *P. acaciicola* as a mycoherbicide on *A. cyclops* would be recommended, provided that sufficient monitoring of treated sites is implemented that primarily focus on the indigenous Fabaceae species. The effective control of *A. cyclops* could be achieved when *P. acaciicola* is used to compliment current mechanical, biological and chemical control methods in an integrated management strategy.

‘n Risiko-assessering van die *Acacia cyclops* terugsterf patogeen, *Pseudolagarobasidium acaciicola*, as ‘n swam-gebaseerde onkruiddoder in die Suid-Afrikaanse strandveld en kalksteen fynbos

Opsomming

Acacia cyclops, ook bekend as rooikrans, is ‘n indringerplant in Suid-Afrika wat oorspronklik vanaf Australië ingevoer is om die sandduine in die Kaap te stabiliseer. Die verspreiding van rooikrans bedreig die sensitiewe biodiversiteit van die strandveld en kalksteen fynbos. Rooikrans terugsterwing is al vir ‘n geruime tyd opvallend in die grootste deel van die plant se verspreiding in Suid-Afrika, alhoewel die veroorsakende organisme, *Pseudolagarobasidium acaciicola*, eers onlangs beskryf is. Hierdie swam is as ‘n geskikte kandidaat vir die ontwikkeling van ‘n biologiese onkruiddoder om rooikrans te beheer, genomineer. Alhoewel die huidige biologiese- en meganiese beheer metodes vir rooikrans gedeeltelik suksesvol is, hou dié indringer steeds ‘n ernstige bedreiging vir die natuurlike ekosisteme in. Die gebruik van ‘n swam-gebaseerde onkruiddoder sal die beheer van rooikrans oor die langtermyn meer koste-effektief maak. Die oorgrote meerderheid van die literatuur wat hersien is, ondersteun die gebruik van swam-gebaseerde onkruiddoders as biologiese beheermiddels, veral as die afname in aanvaarbaarheid en beskikbaarheid van chemiese beheermiddels in ag geneem word. Aangesien die *Pseudolagarobasidium* genus uit saprofiete, opportunistiese fakultatiewe patogene en endofiete bestaan, word daar voorspel dat *P. acaciicola* ‘n soortgelyke biologiese karakter sal hê. Dit is hoogs waarskynlik dat hierdie swamspesie inheems is, alhoewel die verspreiding wyer mag wees as wat oorspronklik voorspel is. Streng maatreëls moet egter steeds in plek wees om te verseker dat nie-teiken plantspesies nie bedreig word nie. Hierdie studie bestaan uit ‘n unieke risiko-analise met verkeie onderafdelings. ‘n Veld-opname is uitgevoer om die siekte-voorkoms van die inheemse houtagtige plantspesies rondom ‘n 100 siek rooikrans plante te bepaal. DNA ekstraksies is vervolgens vanuit die wortels van siek inheemse plantspesies en -rooikrans uitgevoer, om uiteindelik die teenwoordigheid van *P. acaciicola* binne die hout te kon bevestig. Uit ‘n totaal van 2432 inheemse houtagtige plante wat aangeteken is, was 22 (0.9%) siek of dood, terwyl die teenwoordigheid van *P. acaciicola* in 10 van hierdie plante (0.4%), wat

ses spesies teenwoordig, bevestig is. Die teewoordigheid van *P. acaciicola* is ook in 47% van die rooikrans bevestig. Alhoewel *P. acaciicola* moontlik ‘n swak opportunistiese patogeen op ‘n verskeidenheid inheemse plantspesies is, dui die lae verhouding van dooie inheemse plante teenoor gesonde plante in die veld op ‘n lae risiko vir die gebruik van *P. acaciicola* as ‘n biologiese onkruiddoder. Patogenisiteitstoetse is op inheemse plantspesies uitgevoer om ‘n aanduiding van gasheervatbaarheid te verkry. Wond-inokulasies is op ‘n totaal van 30 inheemse plantspesies by twee veldstudie-areas uitgevoer, terwyl wond- en grond-inokulasies op 17 inheemse spesies potplante in die kweekhuis uitgevoer is. Die optimale temperatuur waarby *P. acaciicola* groei, is bepaal om die swam se seisoenale- en habitatsvoorkeure beter te verstaan. Plante van vyf uit die nege inheemse Fabaceae spesies het doodgegaan, terwyl ‘n enkele plant van vier nie-Fabaceae spesies doodgegaan het. Alle plante buite die Fabaceae familie het oorleef in die veld na inokulasie. Slegs rooikranssaailinge het na grond inokulasie doodgegaan. Lengtedeursnee van die stam en wortels van elke geïnokuleerde plant het bevestig dat daar geen sistemiese infeksie in Fabaceae spesies wat inokulasie oorleef het, plaasgevind het nie. Infeksies in vatbare Fabaceae spesies was oor die algemeen meer ernstig as infeksies in vatbare nie-Fabaceae spesies. Die optimale groei van *P. acaciicola* het by 35°C plaasgevind, wat aandui op ‘n voorkeur vir somerstoestande. Inheemse Fabaceae spesies het meer vatbaar as vatbare plantspesies van ander families voorgekom. Hierdie verskynsel dui op ‘n sekere vlak van spesifisiteit, alhoewel daar geen duidelike filogenetiese grense vir vatbare spesies bepaal kon word nie. Behalwe vir die feit dat *P. acaciicola* ‘n fakultatiewe patogeen op rooikrans is, stel resultate van hierdie studie voor dat hierdie swam hoofsaaklik ‘n saprofiet is wat soms ook ‘n opportunisties patogeen op sekere inheemse Fabaceae is en moontlik slegs ‘n swak opportunistiese patogeen op plantspesies buite die Fabaceae familie is. Die swak en oneffektiewe bestuur van rooikrans in hierdie bedreigde plantegroeitipes hou egter ‘n groter bedreiging in as die gebruik van *P. acaciicola* as ‘n biologiese onkruiddoder. *Pseudolagarobasidium acaciicola* word daarom aanbeveel vir die beheer van rooikrans, mits voldoende monitering, wat fokus op inheemse Fabaceae spesies, gepaard gaan met die gebruik van hierdie biologiese onkruiddoder. Rooikrans kan effektief beheer word as *P. acaciicola* ingespan word om huidige meganiese-, biologiese- en chemiese beheermetodes in ‘n geïntegreerde bestuurstrategie te komplimenteer.

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Contents

Chapter 1

Risk assessment of the *Acacia cyclops* dieback pathogen, *Pseudolagarobasidium acaciicola*, as a mycoherbicide in the South African strandveld and limestone fynbos – a review

1.1. Introduction

After being introduced to South Africa in 1835 (Poynton, 2009), the Western Australian tree *Acacia cyclops* A. Cunn ex G. Don (Fabaceae, Mimosoideae), became one of the most invasive tree species in the country. These trees were initially introduced to stabilize the moving sand dunes along the southwestern Cape coast because of their ability to tolerate the blast of the sand, the salt from the ocean and the saline soil (Marcar *et al*., 1995; van Wilgen *et al*., 2011). These trees are now distributed all along a coastal belt in the Western-, Eastern- and Northern Cape provinces of South Africa from Hondeklipbaai to East London (Coates Palgrave, 2002), preferring limestone fynbos and strandveld as habitat (Henderson, 1998). In Australia, the tree is known as the red-eyed wattle or western coastal wattle, while it is known colloquially as "rooikrans" in South Africa.

Rouget and Richardson (2003) found it to be the most widespread weed in the Agulhas Plain and Cape Peninsula, the two areas with the most detailed vegetation maps in the fynbos biome. A potential range expansion was also predicted for *A. cyclops* in these areas. The seeds are known to be fed on and subsequently dispersed by a variety of vectors including redwinged starlings (Fraser, 1990), pied starlings (Török, 1999), European swallows (Hofmeyr, 1989), barn swallows (Underhill and Hofmeyr, 2007), baboons (Richardson and Kluge, 2008), ants and rodents (Holmes, 1990). This invasive tree has become economically important in strandveld and fynbos areas where very few native trees grow. The wood is used as fuel and the profits made in this industry benefit many poor local communities.

1.2. Threats associated with the *Acacia cyclops* invasion

The largest part of *A. cyclops*' distribution lies within the boundaries of the fynbos biome in the Cape Floristic Region. This biome contains more plant species per unit area than any

other biome on earth. With almost 9,000 plant species within an area of around 90,000 km^2 (Manning, 2007), it is essential to control invasive weeds within this biome. Fynbos is known as a fire prone habitat and woody invasive weeds increase the fuel load that consequently lead to hotter fires that kill native seed banks (Brooks *et al*., 2004).

Weeds like *A. cyclops* physically outcompete indigenous plants and their seed banks by forming a dense upper canopy, and blocking light from the undergrowth (Vosse, 2007). Dense stands of this weed also decrease the grazing capacity on stock farms. There is a high probability of losing rare plant and dependent species in the long term if *A. cyclops* is not effectively managed. Higgins *et al*. (1999) found that *A. cyclops* poses the greatest threat to native, rare and endemic species in the Cape Peninsula respectively when compared to other Australian acacias. Australian acacias were found to cause the most significant decreases in indigenous species richness in South Africa (Gaertner *et al*., 2009). Consequently, these invaders significantly alter the natural ecosystems and the services they provide (Le Maitre *et al*., 2011), amounting to an estimate annual cost of around R4 billion on grazing, biodiversity and water resources (de Lange and van Wilgen, 2010). These negatives effects are felt by a range of social classes, including some of the poorest local communities (Kull *et al*., 2011). Furthermore, the reduction of invasive weeds like *A. cyclops* would likely lead to an increase in aesthetic value and possibly higher ecotourism revenue, especially within nature reserves (Higgins *et al*., 1997).

1.3. Invasion hypotheses

The successful or unsuccessful establishment and spread of exotic plants within natural systems can be ascribed to many different key environmental factors. The enemy release hypothesis (ERH) attributes the success of invaders to the lack of enemies in the invaded environment (Keane and Crawley, 2002), while the biotic resistance hypothesis highlights the competitive pressure from indigenous species on exotic species that inhibits invasions (Simberloff, 2010). It also appears as if the lack of generalist enemies in the newly established habitat play as an important role in the process of invasion, if not more important, than the lack of specialist enemies (Halbritter *et al*., 2012). The ERH is not necessarily the sole explanation for non-indigenous species becoming invasive. Agrawal *et al*. (2005) concluded that the escape from one suite of enemies does not automatically give organisms immunity against all enemies, and that invasions are likely the result of the varying effects of enemies through time and space that leads to windows of opportunity. A study by Parker and

Gilbert (2007) also found no significant difference between the attack from herbivores on invasive and native clover species in California's coastal prairies. In a recent study by Bennett and Strauss (2013), invasion is attributed to the lack of responsiveness from non-indigenous species to soil communities varying across landscapes. This allows these plants to establish and spread across a wide range, outcompeting indigenous species that are sensitive to soil community composition. There seems to be a wide and varying range of biotic and abiotic factors contributing to the establishment and spread of each invasive species.

The success of *A. cyclops* as a strandveld and fynbos invader, in all probability, can be ascribed to its high propagule pressure (van Wilgen *et al*., 2011). Biotic resistance can, to some extent, be overcome by propagule pressure of the invading organism (D'Antonio *et al*., 2001). Rooikrans seed was sown in large quantities as part of a vast dune stabilization project undertaken by the government in the southern Cape between 1850 and 1974 (Shaughnessy, 1980). Between 1901 and 1951, the dunes in the Still Bay area were stabilized by planting 445 hectares of *A. cyclops* (Avis, 1989). After a non-indigenous plant species has established itself and enters the invasive phase, which could be decades after its initial introduction, it becomes nearly impossible to successfully implement a short-term eradication strategy (Evans, 2000). Consequently, the development of a long-term control strategy is essential for these species.

1.4. The control of *Acacia cyclops*

Since the realization of the negative effects of *A. cyclops* on local fauna and flora, various methods have been implemented to control this alien invader. According to the Conservation of Agricultural Resources Act 43 (CARA) of 1983, and the more recent National Environment Management: Biodiversity Act (NEMBA), *A. cyclops* is a Category 2 invader. Species within this category may not be planted without a permit and have to be controlled or eradicated if possible on privately owned land by law. This often results in farmers simply burning the invaded land, leading to mass germination of seed in the soil and subsequent further proliferation of species like *A. cyclops* (Munalula and Meincken, 2009).

1.4.1. Non-biological control

Mechanical clearing is a very effective way of eradicating *A. cyclops* mainly because this species has a lower number of sprouting seedlings than most other invasive acacias

(Milton and Hall, 1981). Rooikrans seed, unlike other invasive acacias, mostly germinate within a year or two of production, while the minority stays dormant for extensive periods. Their seed rain is lower (2,000 seeds m^{-2}) than species like *Acacia saligna* (Labill). Wendl. (10,000 seeds m^{-2}) and *Acacia longifolia* (Andrews) Willd. (11,500 seeds m^{-2}), while the density of soil-stored seed is also much lower (5,100 seeds m^{-2}) than their relative *Acacia melanoxylon* R. Br. (49,000 seeds m^{-2}) (Milton and Hall, 1981). This results in more manageable seed banks and consequently a smaller number of sprouting seedlings. Fire has been used to compliment mechanical control by ensuring the destruction of seed, stimulating germination and subsequently also exhausting seed banks (Pieterse and Cairns, 1986; 1988). The temperatures of these fires are too hot for most fynbos seed to survive.

Making mechanical clearing even more effective is the fact that rooikrans does not vigorously coppice after clearing, and needs no chemical application on the tree stump. The problem with mechanical control however is that it is labour intensive and costly, often demotivating farmers from clearing their land of invasive tree species. While mechanical clearing has proven to be a very successful control method for relatively small scale projects, it is very costly to implement on a large scale.

Rooikrans is one of nearly 70 Australian acacias that have been introduced in South Africa (Poynton, 2009). *Acacia cyclops* was predicted to invade a greater area of the Cape Peninsula (up to 64%) than any other invasive weed (Higgins *et al*., 1999). In 1996, Le Maitre *et al*. (2000) estimated that *A. cyclops* occupied more than 45% (291,000 of 643,000 ha) of the total area invaded by Australian acacias in South Africa (measured in closed canopy hectares). However Kotzé *et al*. (2010) estimated that this figure has decreased by 81% to 55,000 ha. These are crude estimates and respective methods implemented by the two authors differ significantly. Although a dramatic drop like this within *A. cyclops* populations in a relatively short period is questionable, any negative or static population growth would likely be attributed to a combination of extensive harvesting and the effect of the biocontrol agents on the trees (van Wilgen *et al*., 2011).

Between 2000 and 2010, Working-for-Water has spent more than R880 million (adjusted to 2010 South African rands) on the clearing of acacias (van Wilgen *et al*., 2011). Although many other factors besides biomass like slope, proximity to sensitive areas and distance to roadside will have an influence on the cost, it would cost approximately up to R15,000 to fell a hectare of closed canopy *A. cyclops*, and in addition have biennial follow-ups for 2 years (Ahmed Khan, Working-for-Water, pers. comm.).

1.4.2. Biological control

When it became clear that mechanical clearing alone is not effectively controlling the spread of rooikrans, research on biological control options commenced. Although it has been recorded that indigenous insects such as tortricid moth larvae (Donnelly and Stewart, 1990) and alydids (Holmes and Rebelo, 1988) can destroy immature *A. cyclops* pods or seeds, the damage they cause have an insignificant effect on the overall reproduction of *A. cyclops*. Research focused on *A. cyclops*' enemies within its natural range, the southwest of Australia.

This search resulted in the release of two biocontrol agents in South Africa. The first is a seed-feeding weevil, *Melanterius servulus* Pascoe (Coleoptera: Curculionidae), introduced in 1991, and the small fluted galler midge, *Dasineura dielsi* Rübsaamen (Diptera: Cecidomyiidae), introduced in 2001 (Impson *et al*., 2004). The latter cause the formation of galls instead of seed pods by laying its eggs within the flower, while the former feeds on and consequently destroys the seed inside the pods. Although both of these Australian insects have been locally successful in reducing reproduction, they do not have a significant effect on the growth rate or mortality of rooikrans (Wilson *et al*., 2011). A variable performance of *D. dielsi* in South Africa has been observed between the levels of infection in different stands of *A. cyclops*. This could possibly be the result of major genetic variability in *A. cyclops* as a result of a complex introduction history, which inhibits recognition by *D. dielsi* (le Roux *et al*., 2011).

The need for a more cost-effective and less labor intensive method to control *A. cyclops* has become apparent. A viable solution to address this large scale problem is the introduction of a biological control agent that would significantly increase the mortality rate of *A. cyclops*.

1.5. Commercial importance of *Acacia cyclops*

South Africa is faced with a major challenge in formulating management strategies for Australian acacias throughout their distribution, recognizing the worth of some species at community- and commercial level, but also the ecological damage they cause in the natural areas where they occur (van Wilgen *et al*., 2011).

Fire wood for fuel is still a fundamental resource for households in poor communities, mainly because it is free and easily accessible (Shackleton *et al*., 2006). The fynbos- and

strandveld environment in which *A. cyclops* has established has little to no trees, resulting in this woody invader being a very important resource for local communities in these areas (Figure 1). Although rooikrans was originally introduced to stabilize sand dunes, the wood is an excellent source of fuel and charcoal and can also be used as durable fencing and screens (Kull *et al*., 2011).

In the Western Cape, rooikrans is rated as the best quality fuelwood, consequently leading to a commercialized industry in some parts of this province where the trees are abundant (Mustart *et al*., 1997; Munalula and Meincken, 2009). Even though the harvesting of the wood is encouraged to assist in the control of the rooikrans, many rural communities have become dependent on this as a source of income, leading to further conflict of interest (Kull *et al*., 2011). Rooikrans also has the potential to be used for gasification and pelletization projects, although these initiatives are fairly marginal due to the cost of moving the cleared plants long distances from invaded areas to processing plants (Guy Preston, Working-for-Water, pers. comm.). Theron *et al*. (2004) estimated the woody biomass (with stem diameters greater than 2.5 cm) of *A. cyclops*, *A. saligna* (Labill) Wendl. and *A. mearnsii* De Wild. in stands where crown cover exceeded 50% on the Cape coastal plains at more than 10,000 tons. Although this indicates the potential for large-scale utilization of the plants, it could lead to environmental degradation and livelihood dependency (Theron *et al*., 2004). Although policies in this country are set to protect economic trade, the value of healthy ecosystems is grossly underestimated. South Africa's Accelerated and Shared Growth Initiative (ASGISA) policy clearly state that economic growth consumerisms will enjoy preference over sustainability and conservation, indicating the broad misconception that conservation is a luxury that can only be attended to once social welfare have been addressed (van Wilgen *et al*., 2011). The fact that the poorest people often rely heaviest on ecosystem services is overlooked. The overwhelming majority of *A. cyclops* harvesters are poor people that are as dependent, if not more so, on the services provided by natural ecosystems as rooikrans wood itself.

The commercial value of *A. cyclops* has restricted the biocontrol programme against it to seed reducing agents that do not damage the non-reproductive parts of the weed (Impson *et al*., 2011; van Wilgen *et al*., 2011). The continuous spread of unwanted *A. cyclops* in coastal areas however has led to this restriction being challenged by biological control researchers (Rouget and Richardson, 2003).

1.6. *Acacia cyclops* dieback

After a noticeable number of rooikrans were seen dying off in the field, the phenomenon was classified as *A. cyclops* dieback. *Acacia cyclops* dieback was first recorded in South Africa in 1969 along the Garden Route and wrongly attributed to a *Ganoderma* species (Taylor, 1969). By the early 1980's dead and dying rooikrans were a common occurrence, especially between George and Still Bay in the Western Cape. These populations are experiencing significant mortality as a result of the dieback disease and it seems as if the disease will eventually spread over the entire distribution area of *A. cyclops* in South Africa (Wood and Ginns, 2006).

1.6.1. Symptoms

The earliest visible sign of stress displayed by affected trees in the field is the discolouration and wilting of the phyllodes. The older leaves tend to shed at an unnatural rate, leaving branches almost bare, with only a few younger phyllodes at the tip of the branch. This is a slow process that can last up to 6 months before the remainder of the phyllodes drop and the aboveground parts die off. A disease interface characterized by dark lines was observed when cutting through the roots of trees at a very early stage of disease. When the trees are at an advanced stage of disease or already dead, a white mycelial mantle covers the roots. This mantle is followed by dry rot that eventually degrades the roots (Wood and Ginns, 2006).

1.6.2. *Pseudolagarobasidium acaciicola*

Initial efforts to isolate the die-back pathogen were unsuccessful, only delivering a leaf pathogen, *Cylindrocladium pauciramosum* Schoch & Crous (Schoch *et al*., 1999), and a root pathogen, *Ganoderma* sp. (Taylor, 1969). After pathogenicity screening tests, neither of these fungi proved to cause mortality in rooikrans. In 1995, an unidentified basidiomycete was isolated from the roots of an *A. cyclops* tree in the early stages of disease in a stand near Vermaaklikheid. This fungus caused 100% mortality of *A. cyclops* in pathogenicity tests (Wood and Ginns, 2006). In 2006, this fungus was named as a new species, *Pseudolagarobasidium acaciicola* (Polyporales, Basidiomycota) Ginns (Wood and Ginns, 2006). *Pseudolagarobasidium acaciicola* is difficult to isolate due to secondary fungi taking over the root system as soon as the tree becomes symptomatic. The pathogen has since been

isolated from the roots of four *A. cyclops* trees along the coastal belt from Hermanus to Still Bay (Wood and Ginns, 2006). The fact that *P. acaciicola* has only been isolated from roots, and that the only internal symptoms visible are in the roots and lower stem, confirms that this pathogen infects the tree via the root system.

1.6.2.1. Taxonomy

Various authors synonymized certain species of the genus *Pseudolagarobasidium* with *Radulodon* because of similar morphological traits (Stalpers, 1998; Nakasone, 2001). This synonymy was rejected when molecular analysis revealed *Pseudolagarobasidium* as a monophyletic group that was well supported (Hallenberg *et al*., 2008). The genus *Pseudolagarobasidium* currently includes six other species, which are *P. pronum* (Berk. and Broome) Nakasone and D.L. Lindner, *P. pusillum* Nakasone and D.L. Lindner, *P. venustum* (Hjortstam and Ryvarden) Nakasone and Lindner, *P. belizense* Nakasone and Lindner, *P. subvinosum* (Berk. and Broome) Sheng H. Wu and *P. modestum* (Berk.) Nakasone and Lindner.

Pseudolagarobasidium acaciicola has only been isolated from South African plant species and is believed to be a native pathogen. The classification of three specimens collected in Kwazulu–Natal by P. A van der Bijl (PREM 602, 669 and 674), which were initially misidentified as *Irpex modestus* Berk., were revised and reclassified as *P. acaciicola* (Nakasone and Lindner, 2012). This finding suggests that *P. acaciicola* occupies a wider geographical range and greater variety of habitats than currently assumed. Another fungus, tentatively identified as *P. acaciicola* (ITS sequence GENBANK AM849050), was isolated from soil in an Indian rainforest. This sequence however differs by 9% and 10% respectively from the two South African sequences (GENBANK DQ517882 and DQ517883) and is very likely to be classified as another species (Nakasone and Lindner, 2012). This relatedness however affirms the complexity of classifying locally occurring fungi as indigenous or non-indigenous.

As the biological character of *P. acaciicola* is still poorly understood, the biological character of its closest phylogenetic relatives might provide some guidelines. According to DNA sequence analyses, *P. belizense* is the closest related to *P. acaciicola*, with both of them being part of the same monophyletic clade along with an undescribed foliar endophyte (ITS sequence GENBANK HM060641) (Nakasone and Lindner, 2012) isolated from healthy cedar mangrove leaves in Thailand (Chokpaiboon *et al*., 2010).

Pseudolagarobasidium pronum is known as a widespread saprobe, but is also the only other species in this genus known to causes a dieback disease, namely that of white lead trees, *Leucaena leucocephala* (Larn.) de Wit, in Western Australia (Wood and Ginns, 2006). Shivas and Brown (1989) associated *P. pronum*, initially misidentified as *P. subvinosum* (Nakasone and Lindner, 2012), with stem and root rot of the same tree species in India. *Pseudolagarobasidium subvinosum*, mainly known as a saprophyte, has however been found to be responsible for a root rot or stem canker of white lead trees in Taiwan (Jang and Chen, 1985) and India (Sankaran and Sharma, 1986). Although white lead trees belong to the same subfamily (Mimosoideae) as *A. cyclops* within the Fabaceae family, recent phylogenetic studies of the Mimosoideae reveal that they are very distantly related within this subfamily (Bouchenak–Khelladi *et al*., 2010; Miller *et al*., 2011; Miller *et al*., 2013). *Pseudolagarobasidium subvisnosum* was re-isolated from the diseased roots of another *Acacia* spesies, *A. decurrens* Willd. and other tree species in Sri Lanka (Petch, 1923). Both *P. pronum* (Sierra Leone) and *P. subvinosum* (Democratic Republic of the Congo) have been reportedly isolated on the African continent. The remainder of the *Pseudolagarobasidium* genus is known as saprophytes (Hallenberg *et al*., 2008; Nakasone and Lindner, 2012). The *P. acaciicola* specimens described by van der Bijl was collected from dead tree stumps (Nakasone and Lindner, 2012), confirming that this species is not only pathogenic but also saprophytic to some extent. With regard to *Pseudolagarobasidium*, it is clear that species within this genus are cryptic and apparently uncommon, although they might be more widespread that currently known and it is likely that there are several species yet to be described.

1.6.2.2. Pathology

Wood and Ginns (2006) tested 42 isolates of various fungal species for pathogenicity on *A. cyclops*. Fungi commonly isolated from *A. cyclops* and screened for pathogenicity included *C. pauciramosum* (11 isolates) and *Fusarium* spp. (five isolates). *Pseudolagarobasidium acaciicola* caused the mortality of all inoculated seedlings and saplings after 2 months in the pathogenicity screening tests. None of the controls died. Purple basidiomata were observed around the dead seedlings on the soil surface. A dark disease interface could be observed in the roots of the dead saplings, along with hyphae occupying the xylem vessels, ray cells and occasionally intercellular spaces. In field trials, trees older

than 10 years were inoculated at two sites near Agulhas National park, resulting in the respective mortality rates of 86% and 54% after 3 years. None of the controls died.

1.6.2.3. Mode of infection

Pseudolagarobasidium acaciicola's exact mode of infection and subsequent cause of pathogenicity on *A. cyclops* are unknown, although above mentioned pathogenicity trials required wounding of the stem to introduce the fungus to the xylem of *A. cyclops*. As a result, the wounding of *A. cyclops* in the field would presumably be a prerequisite for infection to take place. Hyphae dimensions of *P. acaciicola* range between 2 and 4 μm (Wood and Ginns, 2006). This implies that a wound of the same dimensions or larger is necessary for hyphae to penetrate into the xylem vessels. Wounds with these dimensions could easily be created by lesion nematodes, *Pratylenchus* spp. (Castillo and Vovlas, 2007). These nematodes have a wide variety of plant hosts and are known as the most common nematode genus that allows fungi access to roots. Some vascular wilts like *Ophiostoma* are known to inhibit root hair production, alter the permeability of root cells or block the xylem vessels, all subsequently leading to reduced water intake and transport (Martín *et al*., 2005). The reduction in water intake ultimately leads to water stress symptoms (like the discoloration and wilting of leaves) similar to *A. cyclops* dieback.

1.6.3. Implications of *Acacia cyclops* dieback

Since *A. cyclops* is a successful invader in large parts of the coastal plains of South Africa, the biotic resistance hypothesis per se would not apply to the *A. cyclops* dieback. Delayed biotic resistance or the new association hypothesis could be better applied to the situation, where invading species form new relationships with locally occurring species within the area of invasion that could impede invasion success (Mitchell *et al*., 2006). Rout and Callaway (2012) also found that invasive plants outside of their natural range generally tend to interact differently with micro-organisms in the soil compared to indigenous plant species.

Alternatively, the susceptibility of *A. cyclops* could be attributed to enemies from *A. cyclops*' native range arriving in South Africa at a later stage. This would allow a window period of absence in which *A. cyclops* could have experienced a loss of resistance to these enemies (Bossdorf *et al*., 2004). The loss of resistance can be explained by the endophyte-

enemy release hypothesis (Evans, 2008) or the similar evolution of increased competitive ability hypothesis (Blossey and Nötzold, 1995), where the lack of enemies leads to a shift in the plant's resource allocation from defense to vegetative growth, reproduction, maintenance or storage. These hypotheses state that plant species that become invasive outside of their natural range, without the mutualistic endophytes from their natural range, can experience sudden widespread susceptibility when a natural enemy is eventually introduced. *Acacia cyclops* dieback could possibly be associated with the trend of collapsing populations of non-indigenous species after their successful establishment and substantial spread (Simberloff and Gibbons, 2004). This slowing down, stop or even reverse of invasions can likely be attributed to the effect of pathogens (Hilker *et al*., 2005). With regard to the high probability of *P. acaciicola* to be an indigenous pathogen as discussed earlier, the delayed biotic resistance hypothesis is however seen as the more likely explanation of the two for *A. cyclops* dieback.

Pseudolagarobasidium acaciicola could provide a compromise for the conflict-of-interest situation between the beneficiaries of *A. cyclops*' commercial exploitation and the need to control this invasive plant. In a study by de Wit *et al*. (2001) on *A. mearnsii*, another commercially important invader, it was concluded that the most viable economic scenario would be to implement weed-attacking biological control in combination with mechanical clearing in some areas, while commercial growing is continued in other areas.

The natural spread of *P. acaciicola* appears to be slow, which would increase its potential to control *A. cyclops* on privately owned land and nature reserves without affecting *A. cyclops* populations near communities that use this tree as a source of fuelwood and income (Wood and Ginns, 2006). From field observations, *P. acaciicola* also seems to kill randomly within dense stands, creating space for the remaining trees to reach harvestable size and be more accessible (Wood, pers. comm.).

1.7. Weed biocontrol in perspective

According to van Wilgen *et al*. (2011), impact reduction is the only feasible tactic with regards to the management of widespread Australian acacias. This tactic would entail chemical and mechanical control combined with weed biocontrol to reduce density and distribution of these invasive trees. Ten biocontrol agents (nine insects and one fungus) have been released onto ten invasive *Acacia* species in South Africa (Impson *et al*., 2009). Although reproductive feeders like *Melanterius servulus* and *Dasineura dielsii* on *A. cyclops* (as discussed earlier) can reduce the rate of spreading, a high and constant level of damage is

needed over a long period of time before the densities of *A. cyclops* will be affected without the aid of additional control methods (Rouget and Richardson, 2003). In cases where the value of the impact of an invasive species is higher than the benefits, more aggressive biological controls should seriously be considered rather than seed attackers (van Wilgen *et al*., 2011). Taking into account the results that weed biocontrol has delivered; this practice is grossly underfunded (van Wilgen and de Lange, 2011). The effectiveness of biological controls that kill plants has varied from substantial to complete (Zimmermann *et al*., 2004), with the key issues being the ability of commercial growers of the invasive plant to protect their crops and the ability of the agent itself to establish and become effective in the field (van Wilgen et al., 2011). In the case of rooikrans, there are no commercial growers, and the proof of establishment and effectiveness of *P. acaciicola* are evident in the field.

Barton (2012) listed 28 fungal pathogens used as biocontrol agents around the world and contains four South African case studies between 1987 and 1991. The gall forming rust fungus, *Uromycladium tepperianum* (Sacc.) McAlpine, introduced as a classical biological control on *A. saligna* in the fynbos biome, is included. The pathogen managed to establish throughout the entire distribution of its host weed and significantly decreased its spread. After 15 years of post-release monitoring, it was found that *U. tepperianum* had decreased tree density at specific sites between 87% and 98%, with an average annual mortality of 18% during the years monitored (Wood and Morris, 2007). This suggests that the use of a fungal biocontrol on an invasive Australian wattle in South Africa could be successful. The use of plant pathogens as biological control agents continues to gain recognition as an effective way of controlling invasive weeds in agricultural and natural ecosystems. In many cases it has been proven to be a safe, effective and convenient method of control (Charudattan, 2001). The major advantage of using biological control compared to chemical control is its evolutionary stability and subsequent low risk for induction of resistance (Evans, 2000; Sundh and Goettel, 2013), even if the relationship between the agent and the host is newly developed as described in the new associations hypothesis. Biological control offers a more sustainable impact as it is based on co-evolving systems where the control agent or enemy can adapt to changes in host genetics, while chemical herbicides are known to encourage resistance development in target weed populations (Heap, 2014).

1.7.1 Mycoherbicides

Bioherbicides can be described as formulations of an organism's infective propagules in a carrier, which can be applied to target weeds in the same way as a chemical herbicide and cause disease, which ultimately should kill the plant (Morris *et al*., 1999). This differs from classical biological control in that the control organisms used in the formulation are locally isolated and not 'released' as a non-indigenous organism to spread independently. Mycoherbicides are simply fungal bioherbicides, with the infective propagules being either spores or mycelia. Strategies involving the use of mycoherbicides are often referred to as inundative or augmentative biological control, an attractive alternative to classical biological control with regards to predictability (Evans, 2000). The development of mycoherbicides is faced with numerous challenges that include the cost of registration, stability and ease-of-use of the product. The greatest challenge however seems to be proving the field efficacy of a product (Hallet, 2005). Although mycoherbicides still form a minor part of weed management, serious investments are being made in this field as producers are forced by the public, research development and environmental degradation to move away from chemical control.

Stumpout® is an example of a registered mycoherbicide that has been successfully used on invasive *Acacia* species in South Africa. The active ingredient of this mycoherbicide is the spores of a wood rotting fungus, *Cylindrobasidium laeve* (Pers.) Chamuris in an oil suspension that is applied to the cut stumps of *Acacia mearnsii* (black wattle) or *Acacia pycantha* Benth. (golden wattle) to kill the developing shoots (Morris *et al*., 1999). Although this fungal species is known as a saprophyte from North America (Nakasone, 1993), it was found naturally colonizing dead *A. mearnsii* stumps near George in the Western Cape Province of South Africa. Both *C. laeve* and *P. acaciicola* belong to the class Agaricomycetes.

Stumpout® is only the second mycoherbicide in the word to be registered for the biocontrol of a tree weed after Biochon®, a product registered in the Netherlands. Biochon®, a formulation of *Chondrostereum purpureum* (Pers.: Fr.) Pouzar, is applied to the cut stump of various hardwood species to inhibit sprouting by promoting wood decay (de Jong, 2000). Subsequently, *C. purpureum* was also registered in Canada and the United States of America as Chontrol® on hardwood tree species including white birch, red alder and aspen (Boyetchko *et al*., 2009). In Lithuania, it has recently been proven that *C. purpureum* is as effective in the

stump treatment of the invasive *Acer negundo* L. (box elder) as the popular chemical herbicide, Roundup®BIO (Lygis *et al*., 2012).

Silverleaf disease of fruit trees is a well known disease caused by *C. purpureum* and therefore a thorough risk assessment was undertaken prior to registration of Biochon® in the Netherlands. The study revealed that the mycoherbicide can effectively control the invasive shrub *Prunus serotina* Erhr. (black cherry) in native forests, without being a risk to the fruit industry, provided inoculations take place at least 5 km from fruit orchards (de Jong, 1988).

Another South African mycoherbicide, Hakatak® (*Colletotrichum acutatum* J.H. Simmonds), was provisionally registered on an invasive shrub *Hakea sericea* Schrad. and J.C. Wendl. (silky hakea), in 1990, but has not since been registered due to a lack of large scale demand. The mycoherbicide was originally applied to seedlings as gluten granules with a mycelial coating. More recently though, a suspension of dried conidia in water have been produced by the PPRI to supply a small, but growing need for this mycoherbicide (Morris *et al.,* 1999). These are good examples of the low risks associated with the development of mycoherbicides from locally occurring fungi with proven field efficacy (Morris *et al.*, 1999). The limited demand for mycoherbicides with single target species could however become an obstacle in the development and production of a registered mycoherbicide.

Mycoherbicides have been used in combination with chemical agents as a form of integrated management. An example of this is the control of *Euphorbia heterophylla* Linn. (wild poinsettia) in Brazil by using the leaf-spot fungus, *Lewia chlamidosporiformans* Vieira and Barreto, in conjunction with the herbicide, fomesafen. By combining the latter herbicide with the fungal pathogen, Nechet *et al*. (2008) developed an effective method to control all three weed populations in their study.

Pseudolagarobasidium acaciicola has been nominated as an appropriate candidate for development as a mycoherbicide to control rooikrans due to its proven pathogenicity and local presence (Wood and Ginns, 2006). A number of formulations and methods of inoculation have been explored to produce a cost effective way of controlling *A. cyclops* with *P. acaciicola*. A suspension of mycelia and water proved to cause high mortality (95–100%) in wild rooikrans trees (Impson *et al*., 2011). The suspension was applied by means of an automatic dispenser into several wounds made by a chisel at the base of the tree. Although some of these trees took up to 6 years to die, a number of adjacent uninoculated trees have also started dying (Wood, pers. comm), indicating *P. acaciicola*'s ability to effectively, albeit slowly, spread from inoculated trees which supports *P. acaciicola*'s development as a mycoherbicide.

1.7.2 Potential risks associated with mycoherbicides

Biocontrol agents that reduce seed production are the only ones that should be used when the weed is of commercial value (van Wilgen *et al*., 2011). This however refers to "classical" biological control agents that are introduced into South Africa and not potential mycoherbicides produced from locally occurring fungi. With the most recent legislation requiring land-owners to co-ordinate an extensive and clear strategy for the control of key invasive species like *A. cyclops* on their land (van Wilgen *et al*., 2011), a mycoherbicide like *P. acaciicola* would have the potential to play a crucial role in long term weed control strategies.

The primary concern for using *P. acaciicola* as a mycoherbicide is the potential threat to native species in the diverse Cape Floristic Region. Indigenous plant species closely related to the invasive plant are most likely to be at risk (Pemberton, 2000), thus species within the Fabaceae family could be susceptible to *P. acaciicola*. Wood (2001) concluded that *P. acaciicola* causes mortality in the seedlings of indigenous legumes *Aspalathus linearis* (Burm. f.) Dahlg. (rooibos), *Crotalaria capensis* Baker (Cape rattle-pod) and *Virgilia oroboides* (Bergius) Salter (keurboom) after inoculations. Although these species share a partial distribution overlap with *A. cyclops*, they rarely occur in the same habitat (Coates Palgrave, 2002). Keurboom is a pioneer species susceptible to a whole suite of pathogens because of its meager investment in defense mechanisms (Coates Palgrave, 2002). In a recent study by Machingambi (2013), stem cankers, root diseases and rot, bracket fungi, rapid wilting and death were all recorded on keurboom. These were caused by *Fusarium* species, *Phomopsis* species, *Armillaria mellea* (Vahl) Quel., *Schizophyllum commune* Fries, *Ceratocystis tsitsikammensis* Kamgan and Jol. Roux and *Ophiostoma plurianulatum* (Hedgc.) H.P. Sydow. *Pseudolagarobasidium acaciicola* was not implicated in any observed disease occurrence of keurboom and appears not to be a natural host of the fungus.

The reason for the mortality of indigenous species in pathogenicity trials by Wood (2001) and not in the field could be explained by the unnaturally large entry wounds caused by artificial inoculations that create direct contact between the plant's xylem vessels and the pathogen (Wood pers. comm.). This allows the pathogen to bypass some of the host's natural defense mechanisms, which includes the production of sufficient resin to protect a wound from being penetrated. Another explanation could be that, even though the pathogen only forms a canker due to host defense mechanisms, the seedling stems are too thin to survive the

canker. This canker would not kill off mature trees in the field and, because field infection takes place via the roots, seedlings should still survive, even though they might lose the infected roots (Wood pers. comm.).

Deliberately increasing the population of an alien pathogen, compared to a native pathogen, could pose a greater risk to the natural environment due to the lack of co-evolution and resistance. Since there are no ITS sequences of *P. acaciicola* submitted from outside South Africa (Wood and Ginns, 2006), it is likely to be an indigenous fungus, with subsequent lower associated risks compared to alien fungi.

1.8 Aims of this study

This project addresses the control of *A. cyclops* through the application of *P. acaciicola* as a mycoherbicide. The main objective, that the following chapters will discuss, is an estimation of the risk involved with using *P. acaciicola* as a biocontrol agent in Strandveld and Limestone fynbos vegetation. The assessment should reveal whether *P. acaciicola* could be detrimental to native species within these vegetation types, especially those in the Fabaceae family, by means of field observations, supported by nursery and field pathogenicity trials. Since the potential of *P. acaciicola* as a mycoherbicide against rooikrans has already been demonstrated (Wood and Ginns, 2006); this study aims to provide evidence as to whether this potential should be realised, and the fungus made available for public use as a mycoherbicide.

1.9 Conclusion

The spread of *A. cyclops* is a major threat to the fragile biodiversity of the fynbos biome. The majority of the literature reviewed supports the use of mycoherbicides as biocontrol agents, especially when taking into account the decrease in acceptance and availability of chemical control agents. Classical biological control efforts on *A. cyclops* are partially successful; however the introduction of a mycoherbicide would increase the cost effectiveness of controlling this weed in the long term. Considering the *Pseudolagarobasidium* genus consists of saprobes, opportunistic facultative pathogens and endophytes, *P. acaciicola* is predicted to have similar biological characteristics. The species is highly likely to be indigenous, although with a wider distribution range than previously envisaged. Strict precautions should however be taken to ensure that non-target plant species

will not be threatened. Field observations in previous studies indicate that *A. cyclops* populations around communities benefiting from its harvest should not be significantly affected when *P. acaciicola* is applied as a mycoherbicide in other areas where the weed is unwanted. Ensuring that *P. acaciicola* becomes available as a mycoherbicide for *A. cyclops* in the future would be beneficial to farmers, alien clearing organizations like Working-for-Water and especially the conservation sector in the Western Cape of South Africa.

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1.11 Figures

Figure 1. The commercial importance of *Acacia cyclops* is evident in the poor community of Melkhoutfontein, near the Still Bay field site used in this study.

Chapter 2

Host range- and molecular detection of *Pseudolagarobasidium acaciicola* in the field

2.1 Abstract

The rich biodiversity of the fynbos biome along the southern coast of South Africa is threatened by the spread of *Acacia cyclops*. A locally occurring root pathogen is responsible for an *Acacia cyclops* dieback disease. *Pseudolagarobasidium acaciicola*, the causal organism, is being considered as a potential mycoherbicide against *A. cyclops*. A risk analysis to determine the effect that *P. acaciicola* could have on the surrounding indigenous plants was undertaken. A field survey was performed to record disease incidence among indigenous woody plant species around 100 diseased *A. cyclops* trees. Subsequently, DNA extractions were made from the roots of the diseased indigenous plants and *A. cyclops* trees to verify the presence of *P. acaciicola*. Of the 2432 indigenous woody plants observed, 22 (0.9%) were dead or dying, and *P. acaciicola* was detected in 10 of these (0.4%) representing six species. *Pseudolagarobasidium acaciicola* was detected in 47% *A. cyclops* trees. Although *P. acaciicola* could be a secondary pathogen, endophyte or weak pathogen in a broad range of indigenous plant species, the extremely low disease incidence is an indication of a low level of risk associated with using *P. acaciicola* as a mycoherbicide.

2.2 Introduction

Acacia cyclops A. Cunn. ex G. Don. (Fabaceae, Mimosoideae) is an invasive weed along the southern coast of South Africa. The tree is of commercial importance for poor local communities selling firewood as a source of income. For this reason, only biological control agents that damage the reproductive parts of the tree have been released. These include a seed-feeding weevil, *Melanterius servulus* Pascoe (Coleoptera: Curculionidae) and a galler midge, *Dasineura dielsi* Rübsaamen (Diptera: Cecidomyiidae) (Impson *et al*. 2004). Although both of these Australian insects have been locally successful in reducing reproduction, they do not have a significant effect on the growth rate or mortality of rooikrans

(Wilson *et al*., 2011). A study on the spread of another commercially important invasive *Acacia* in South Africa concluded that the implementation of a weed-attacking biological control to compliment mechanical control would be the most viable and economic solution to the spread of the species (de Wit *et al*., 2001).

Acacia cyclops dieback is a common occurrence throughout the largest part of the weed's distribution range since the 1980's. The causal pathogen, *Pseudolagarobasidium acaciicola* (Polyporales, Basidiomycota) Ginns, was isolated from *A. cyclops* roots and tested for pathogenicity on *A. cyclops* seedlings in glasshouses (Wood and Ginns, 2006). This testing and field observations provided support for the development of this locally occurring pathogen as a bioherbicide. Prior to mass production and of this pathogen and its release, an in-depth risk analysis is needed to provide an indication of the risk levels that indigenous plants in the species rich fynbos biome could be exposed to. Determining the relationship between *P. acaciicola* and indigenous plant species will provide a better understanding of the potential risks associated with using this fungus as a mycoherbicide on *A. cyclops*. Although the order Polyporales are generally characterized as saprophytic fungi on wood and litter (Hibbet and Donoghue, 1995), the genus *Pseudolagarobasidium* also hosts facultative and opportunistic pathogens (Nakasone and Lindner, 2012) as well as endophytes (Hallenberg *et al*., 2008).

Although numerous studies have assessed the risk of exotic fungi as classical biocontrol agents, few discuss the risks associated with the use of locally occurring fungi as mycoherbicides. Sundh and Goettel (2013) state that the extent of the actual increased exposure of non-target plants to mycoherbicides is an issue that needs to be further investigated. The application of mycoherbicides, or augmentation biocontrol, pose a relatively lower risk when compared to classical biological control, given that the fungus used in the formulation is indigenous to the area of intended use (Sundh and Goettel, 2013). The potential risks include the spread, population establishment and adverse effects of the fungus on non-target organisms outside the area of intended use. Another risk to consider is the influence that a higher than normal concentration of a fungal species might have on the soil community (Cipriani *et al*., 2009). The degree to which *P. acaciicola* can move and persist in the soil is uncertain. All of the literature reviewed during this study associated a low environmental risk with the use of mycoherbicides. Even if harmful effects arise from the application of a mycoherbicide, the effects are expected to cease after the discontinuation of inoculations and the subsequent return of population numbers to a background level (Sundh and Goettel, 2013).

The fact that *P. acaciicola* is already present in the field implies that an adapted risk assessment should be considered rather than the traditional assessment of host specificity testing for classical agents. The methodology of a post-introduction assessment is a more appropriate guideline for this study. According to Louda *et al*. (2003), the physiological host range can largely be determined by testing host-specificity, but proves to be inadequate when predicting the ecological host range. The reason for this is that a multitude of factors contribute to host selection in the field. These factors can include dispersal patterns of the host and the biocontrol agent, a variation in life history and even the type of habitat. A suggested methodology to follow for a post-release assessment by Carvalheiro *et al*. (2008) consists of two components. The first is to describe the food web in the invaded habitat by studying the trophic relations between herbivores, plants and parasitoids. The second component focuses on the effect that the abundance of the invasive weed and the biocontrol agent have on the surrounding indigenous populations of species by means of statistical testing.

For this study, the most important trophic relations are between *P. acaciicola* and the indigenous woody vegetation that act as potential hosts. These relations along with the effect the fungus has on the indigenous plant species should be reflected by the number of dead or dying native plants surrounding dead *A. cyclops* trees. Although this would give an indication that the biocontrol agent might be a threat to the native ecological community, the only way to prove this would be to isolate *P. acaciicola* from the plant tissue. Isolating *P. acaciicola* from rooikrans roots is however challenging due to the high frequency of other secondary fungi present (Wood, pers. comm.).

To date, very few isolations from diseased *A. cyclops* successfully delivered *P. acaciicola* (Wood pers. comm.). Successful isolations were all made from the roots of trees with early symptoms. By the time the trees begin dying back secondary fungi like *Ganoderma* species have started colonizing the root, making it difficult to associate *P. acaciicola* with the dieback (Wood and Ginns, 2006). A more accurate approach would be to develop a species–specific primer and amplify DNA from *A. cyclops* root tissue to verify the presence of the pathogen.

Verifying the presence of a fungus within woody plant material by means of DNA extraction can be challenging. DNA extraction methods used for root samples are not only time consuming, but they also tend to yield impure DNA in low quantities as a result of phenolics, polysaccharides and other secondary metabolites contaminating the DNA in the samples (Khan *et al*., 2007). However, a number of studies have managed to accurately verify

the presence of fungal species within woody plant material through the optimization of molecular techniques (Ridgeway *et al*., 2002; Retief *et al*., 2005).

One of the few protocols designed around fungal pathogens in hard woody root material was developed by Retief *et al*. (2005) for the molecular detection of *Phaeomoniella chlamydospora* Crous and Gams in grapevine wood. Before the latter study, DNA extraction and amplification of *P. chlamydospora* was successful from fungal cultures by using a variety of protocols (Doyle and Doyle, 1987; Lee and Taylor 1990; Groenewald *et al*., 2000; Ridgeway *et al*. 2002), but less successful from the grapevine wood samples. Retief *et al*. (2005) ultimately combined and optimized the protocols of Lee and Taylor (1990) and Ridgeway *et al*. (2002) who produced successful amplification from the wood samples.

The first objective of this chapter was to quantify the occurrence of dead or dying indigenous woody plants (DDIPs) around *A. cyclops* trees displaying dieback symptoms in the field. Secondly, the presence of *P. acaciicola* within the DDIPs and *A. cyclops* trees displaying dieback symptoms was to be determined by means of a species–specific primer to give an indication as to whether *A. cyclops* is the exclusive host of *P. acaciicola* in the field. A taxonomic assessment of the DDIPs were undertaken to determine whether possible susceptible species can be circumscribed. Furthermore, the roots of symptomatic plants were investigated macro– and microscopically to provide possible explanations for the pathogenicity of *P. acaciicola*. The results of this chapter ultimately form the basis of the risk assessment.

2.3 Materials and methods

2.3.1 Field survey

Two study sites were selected within the distribution range of *P. acaciicola* in the areas surrounding Still Bay and Walker Bay (Hermanus) (Figure 1). The study sites included a wide range of plant species and excluded any extremely dense stands of *A. cyclops*, as little or no native vegetation grows within these stands. Dead or diseased *A. cyclops* trees at each site were identified, and within a 3 m radius of each *A. cyclops* tree, the number of indigenous woody plants was recorded (Figure 2). This ensured that the largest area of root occupation is included around each tree and includes the potential dispersal distance of *P. acaciicola* via the roots. Indigenous plant species were analysed around a total of 100 *A. cyclops* trees. The indigenous plants were visually classified as either healthy or dying/dead.

The roots from all DDIPs and rooikrans trees that formed part of the survey were excavated and stored at 8°C for no longer than 5 days, before being processed for DNA extraction. All the native plants that formed part of the survey were photographed, coded and subsequently identified to species or subspecies level (Manning, 2007; Bohnen, 1986, 1995; Mouton and Naudé, 2008; Oberholzer, 2010; Mustart *et al*., 1997; Moriarty, 1997; www.ispot.org.za).

2.3.2 Sample preparation

To prepare the samples for DNA extraction, thin disc sections of approximately 3 mm wide were made from the root sample after thorough rinsing with water. These discs were cut from various parts of the root and crown, especially where the disease could easily be observed. After the bark was removed, discs were sterilized by submergence in ethanol (98%) for 15 seconds. They were allowed to dry before being cut up into fine pieces with a flame-sterilized pruner. Before being manually ground down to shavings with a Husqvarna® reliance hand mill, some of these pieces were cut into 2 mm^3 cubes and plated out on potato dextrose agar with streptomycin (PDA^+) in an attempt to isolate *P. acaciicola* (both before and after ethanol sterilization). Isolations were attempted from the roots of all DDIPs and 20 *A. cyclops* trees. Ethanol (96%) was used to sterilize the mill between samples. The shavings were milled with an IKA® A11 basic analytical mill and sieved (600 µm) to produce a fine powder that was stored at -80°C. The analytical mill was sterilized with soapy water and 96% ethanol between each sample.

2.3.3 DNA extraction

A variety of DNA extraction protocols were performed from the wood powder of inoculated *A. cyclops* trees that included a modified soil DNA extraction protocol (Cullen *et al*., 2001), Zymo Plant/Seed DNA Kit™ (Zymo Research, Orange, California, USA), DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA), Phire® Plant Direct PCR Kit (Finnzymes Oy, Finland) and a DNA extraction protocol by Retief *et al*. (2005). DNA concentrations were determined using a Nanodrop™ ND–1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). DNA from the wood powder of dead or dying *A. cyclops* was subsequently extracted using the protocol that delivered the highest DNA concentration from

the powder. After optimization, a modified version of the protocol used by Retief *et al*. (2005) was used.

From each sample, two 2–mL Eppendorf tubes were filled with 100 mg of the root powder and 1 mL 2% CTAB buffer (0.2 M TrisHCl pH 8.0.; 1.4 M NaCl; 0.02 M EDTA) was added. Sterile glass beads were added and the tubes were shaken in a Retch mill for 5 minutes at 30 Hertz. The tubes were spun down and placed in a water bath at 65°C for 30 minutes, inverted and placed back in the water bath for another 30 minutes. After removal from the water bath, 400 µL of cold chloroform: isoamylalcohol (IAA) (24:1) was added to each tube and vortexed. The tubes were centrifuged for 15 minutes at 13,000 revolutions per minute (rpm). The resulting supernatant was transferred into new tubes and the steps following the water bath were repeated. Each new tubes with supernatant received 50 µL of 7.5M ammonium acetate (NH_4OAc) and 600 µL of cold isopropanol.

After an hour of incubation at -20°C, the tubes were inverted 10 times and centrifuged for 10 minutes at 14,000 rpm. The supernatant was discarded and the remaining pellet was dried for 5 minutes and resuspended in 500 µL of TE_{01} buffer. Another 500 µL of cold chloroform–IAA was added and centrifuged for 5 minutes at 14,000 rpm. The supernatant was transferred to new 2–mL Eppindorf tubes. The steps after adding the TE_{01} buffer were repeated, after which 50 µL of 3M NaOAc and 1 mL 100% ethanol were added. After incubation for an hour at -20°C, the tubes were centrifuged for 10 minutes at 14,000 rpm. The supernatant was discarded and the tubes were left for 5 minutes. Two hundred µL of 70% ethanol was added to each tube and centrifuged for 5 minutes at 14,000 rpm. The supernatant was discarded and tubes were placed in a heating block for 30 minutes at 60°C. The pellet was resuspended in 50 µL TE_{01} buffer and stored at -20°C for a maximum of 24 hours before use. After use, DNA was stored at -80°C.

2.3.4 Polymerase chain reaction for *P. acaciicola* species identification

In this study, species–specific forward and reverse primers, Pac1 (5'-ATGACAGGGTTGTTGCTGGCCC-3') and Pac2 (5'- GGGCGCAAGGTGCGTTCAAAG-3') were designed for the South African *P. acaciicola* sequences DQ517882 (CBS 115544, PPRI 7336) and DQ517883 (DAOM 230979, CBS 115543, PPRI 7335) retrieved from the National Centre for Biotechnology Information's nucleotide database. Primers were designed using Primer3 (version 4.0.4). This enabled sequencing of all the root tissue samples to test for the presence of *P. acaciicola*. A polymerase chain reaction (PCR) was performed with a

GeneAmp® PCR System 2720 thermocycler (Applied Biosystems, Foster City, California, USA) in a total reaction volume of 40 µL. Each reaction contained 1 x PCR buffer (Bioline, London, United Kingdom), 0.2 µM of each primer, 0.2 mM of dNTPs, 2 mM $MgCl_2$, 0.05 mg/ml bovine serum albumin (BSA) Fraction V (Roche Diagnostics South Africa, Randburg, South Africa) and 0.65 U BIOTAQ™ (Bioline, London, UK). The PCR was performed using the following conditions; an initial denaturing step of 3 minutes at 95°C, 35 cycles of denaturing for 1 minute at 95°C, annealing for 1 minute at 58°C and extension for 1 minute 30 seconds at 72°C. This was followed by a final extension at 72°C for 5 minutes. A negative control (deionised, autoclaved water) was included in each PCR reaction.

PCR products were resolved in 1% agarose gels for electrophorosis. A solution of ethidium bromide (1 µL) was added to the agarose to stain DNA fragments in order to visually compare it against a 100 base pair DNA ladder (Thermo Scientific). All DDIPs that amplified were then sequenced (DNA Sequencing Unit, Central Analytical Facility (CAF), Stellenbosch University) using the BigDye Terminator version 3.1 sequencing kit (Applied Biosystems) to confirm the presence of *P. acaciicola*. All indigenous plant samples and (due to time and cost restrictions) 20 representative *A. cyclops* samples that amplified, were sent to the CAF for sequencing.

2.3.5 Symptom observations

Cross sections of the dead or dying *A. cyclops* crown and roots were photographed to give an indication of *P. acaciicola*'s movement through the plant. Microscopic photograhps were taken of thin transverse sections cut from the stems of both inoculated *A cyclops* trees and *A. cyclops* trees showing symptoms in the field. The sections were stained with analine blue in lactophenol for 2 minutes and mounted in glycerol to be examined with a Zeis Axioskop light microscope.

2.4 Results

2.4.1 Field survey

In total, 2432 individual indigenous woody plants were surveyed representing 85 species from 27 families (Table 1). The survey included five indigenous plant species belonging to the Fabaceae family making up 4.9% of the total of individuals. Of the 2432

woody plants, 22 (0.9%) were diseased or dead (Table 2). These DDIPs represented 11 different species from eight families. None of the indigenous Fabaceae species were found to be diseased or dead in the field. Outside the 3–m radii it was observed that indigenous plants appeared to be in a healthy state, even where more than approximately 50% of *A. cyclops* trees within the area had died. Field sites where approximately more than half the *A. cyclops* trees have died were common. None of the DDIPs and only a single *A. cyclops* plant yielded *P. acaciicola* through isolations, although *Ganoderma* and *Trichoderma* species were common. The *P. acaciicola* strain was isolated from the roots of a dead *A. cyclops* tree at Still Bay.

2.4.2 Molecular detection

During gel electrophoresis (Figure 3), 77 of the 100 *A. cyclops* and 15 of the 22 DDIPs produced bands using the designed primer pair. After sequence analysis, 10 of the 22 (45%) DDIPs tested positive for the presence of *P. acaciicola* in their roots. These plants are listed in Table 2 and comprise six different plant species from four families. *Pseudolagarobasidium acaciicola* was detected in 12 of the 20 (60%) *A. cyclops* samples that were sequenced. This translates to 47% when extrapolating this figure to the 77 *A. cyclops* samples that amplified during electrophoresis. The majority of sequences obtained that were not *P. acaciicola* were identified as *Ganoderma lucidum* (Leyss. ex Fr.) Karst by performing a BLAST search.

2.4.3 Symptom observations

Typical symptoms caused by *P. acaciicola* as described by Wood and Ginns (2006) were observed in the root sections of nearly all diseased or dead *A. cyclops* samples (Figures 4 and 5). Although aboveground symptoms of some the DDIPs resembled that of *A. cyclops* dieback (leaf senescence and wilt), none of the typical symptoms could be observed in the root sections of these plants. Disease symptoms observed on the stem of *Polygala myrtifolia* L. (thin fingerprint-like black lines) resembled symptoms on *Protea* species caused by *Phytophthora cinnamomi* Rands (Tammy Jensen pers. comm., USPP disease clinic). In the case of *Chrysanthemoides monilifera* (L.) T. Norl., *Muraltia spinosa* (L.) F. Forest and J. C. Manning and *Passerina corrymbosa* Eckl. Ex C. H. Wright, the plants were already dead and

the wood at an advanced stage of decay, which disguised any possible root symptoms. The roots of the other DDIPs appeared asymptomatic.

The margins of the lesions on symptomatic *A. cyclops* roots were defined by irregular thin black lines that separate the healthy root tissue from the dead wood. Although less often, some lesions were clearly sectorial (Figure 4a and b), especially infected lateral roots. Most lesions also had a watery appearance. In the majority of the sections, the rot could be observed in the heartwood, with a narrow corridor joining the white rot to the vascular cambium (Figure 4iii), from where it progressively surrounds the sapwood (Figure 4c and d). Other cases involved rotting sapwood without the heartwood being affected (Figure 4e). In the majority of the infected *A. cyclops* crowns the rot of the xylem seem to precede the dying of the heartwood. The rot also varied from partial cover of the section surface area (Figure 4a-d) to near complete cover (Figure 4e and f). Large volumes of the smaller lateral roots were infected and this appeared to serve as a good source of infection for the larger taproot. In some instances, infection into the taproot was clearly visible from one or more of the lateral roots (Figure 5a). Lengthwise sections clearly display the girdling of the heartwood by the rot due to the movement of the pathogen through the vascular tissue (Figure 5b). Microscopic observations of the vascular cambium prove that *P. acaciicola*'s hyphae primarily move up and down the larger xylem vessels, but do not completely block the plant's nutrient transport (Figure 6a). Purple basidiomes (Figure 6b) were observed in the phyllode litter at the base of some of the mature inoculated rooikrans.

2.5 Discussion

The field survey revealed a low fraction (0.9%) of DDIPs with the majority of plants appearing healthy. Taking into account that the majority of the survey was performed in areas where more than 50% of the rooikrans were dead or dying, the low number of DDIPs indicates that increased *P. acaciicola* populations by use as a mycoherbicide should pose a low risk to the indigenous fynbos and strandveld species. Within the fraction of DDIPs, abiotic factors and other disease causing organisms could have contributed to the death of these plants. Although *P. corymbosa* represented approximately one third of the DDIPs, this member of the Thymeleaceae family is known to be a short-lived pioneer species, typically dying off in fynbos or strandveld communities that are between 10 and 15 years old (Oliver, 2006).

Sequencing results verified the presence of *P. acaciicola* in almost half of the diseased *A. cyclops* and approximately the same portion of the DDIPs. It should however be kept in mind that proving the presence of a fungal species within a plant species does not necessarily imply that the fungal species concerned is the direct cause of the plant's death or disease, as it may be either a secondary organism or a non-lethal endophyte living in a parasitic relationship with the indigenous plant species. This possibility is confirmed by the majority of species within the *Pseudolagarobasidium* genus being saprophytic, with the remainder being either endophytes or facultative weak pathogens (Nakasone and Lindner, 2012; Hallenberg *et al*., 2008). Koch's postulates by means of inoculation are crucial in confirming the pathogenicity of a species.

Confirming the presence of *P. acaciicola* in indigenous plant species does however give an indication of the host range of this fungal species. This is the first record of *P. acaciicola* being detected in a wild plant species other than *A. cyclops*, although no isolations were successful. The two Polygalaceae-, two Thymeleaceae-, one Ebenaceae- and one Proteaceae species within which *P. acaciicola* was detected are not phylogenetically related, providing evidence that *P. acaciicola* has multiple hosts. Three possible theories regarding *P. acaciicola*'s biological character can potentially be inferred.

For one, *P. acaciicola* could be a broad pathogen, affecting a large number of plant species from different families. To support this theory, the susceptibility of the DDIPs that tested positive for *P. acaciicola* needs to be proven by field or nursery pathogenicity trials (Chapter 3). This theory would imply that *P. acaciicola* was in fact the cause of death or disease for the above mentioned DDIPs. Most of the DDIP species were well represented in the overall survey, but with a very low rate of mortality or disease. For this reason, assuming *P. acaciicola* acts as a pathogen, the field survey would support the classification of *P. acaciicola* as a mild pathogen (and not an aggressive pathogen) on certain indigenous species. *Pseudolagarobasidium subvinosum* has also been associated with disease symptoms of a wide range of plant hosts (Nakasone and Lindner, 2012). Plant species susceptible to *P. acaciicola* could share phylogenetically, morphologically or physiologically traits with *A. cyclops*.

The second possibility would be that, although *P. acaciicola* is an opportunistic pathogen on rooikrans, it functions as a saprophyte on some indigenous species, explaining the presence of *P. acaciicola* on the DDIPs. This would mean that *P. acaciicola* was not the primary cause of death or disease on the DDIPs, but colonized the dead wood as a saprobe. The saprophytic nature of *P. acaciicola* is strongly supported by three collections of this

species by P. A van der Bijl (PREM 602, 669 and 674) misidentified as *Irpex modestus* Berk (Nakasone and Lindner, 2012). These specimens were all isolated from a dead tree stump in Kwazulu–Natal, South Africa. In addition, the indigenous plant species in which *P. acaciicola* was detected were already dead, with some of the roots at an advanced stage of disintegration.

The third and final possibility suggests that *P. acaciicola* has an endosymbiotic relationship with some of the fynbos and strandveld species. Mutualistic relationships between endophytes and woody plants are uncommon (Faeth and Fagan, 2002), implying that is more likely that the relationship between *P. acaciicola* and the indigenous plant species, if *P. acaciicola* is in fact an endophyte, can be described as a commensalism, parasitism, or a combination of the two. Some endophytes can also be latent pathogens (Evans, 2008), implying that *P. acaciicola* may occasionally switch from being an endophyte to a pathogen. Various fungal species within close phylogenetic proximity to *P. acaciicola* have been isolated from healthy mangrove leaves in Thailand (Chokpaiboon *et al*., 2010) and cacao stems in Cameroon (Crozier *et al*., 2006). This theory also suggests that *P. acaciicola* was not the primary cause of death of the DDIPs in which it was detected and that *A. cyclops* is the only susceptible species of this endophyte. The widespread occurrence of the *A. cyclops* dieback could be explained by indigenous plants acting as alternative hosts and sources of inoculum.

The potential opportunistic nature of *P. acaciicola* is an example of a recent trend during the last two and a half decades of fungal pathogens emerging in non-native *Acacia* species. *Ceratocystis fimbriata* Ellis and Halstead s.l. was recorded on *Acacia decurrens* Willd. in Brazil, causing a canker and subsequent death of these trees (Ribeiro *et al*., 1988), while another novel *Ceratocystis* species, later named *C. albifundus* Morris, de Beer and M.J. Wingfield (Wingfield *et al*., 1996) was found to cause the wilt and death of *A. mearnsii* in South Africa (Morris *et al*., 1993). Native pathogens can play an important role in limiting the spread of invasive weeds (Duncan and Williams, 2002; Beckstead and Parker, 2003).

There seems to be a strong association of *Ganoderma* species with *A. cyclops*. Wood and Ginns (2006) consistently isolated a *Ganoderma* species from *A. cyclops* roots, although none of the 14 isolates caused mortality in pathogenicity tests. Even with the first report of the *A. cyclops* dieback, a *Ganoderma* species was thought to be the responsible pathogen (Hall, 1979). *Ganoderma* species are known as wood decaying fungi, normally saprophytic or parasitic on dead or living tree stumps (Keypour *et al*., 2010). *Ganoderma lucidum*, detected in some sequenced samples of *A. cyclops* and DDIPs, has been isolated in Western India,

causing root rot and subsequent mortality of *Acacia* and *Prosopis* trees (Bhansali, 2012). It was one of the most frequently encountered fungi in natural agroforestry stands in this part of India. Bhansali (2012) found that once a tree is wounded, *Ganoderma* rapidly colonizes the outer wood or sapwood. An undescribed species similar to *G. lucidum* has also been reported to be pathogenic on certain broadleaf tree species in Queensland, Australia, invading the xylem and phloem tissue in some cases (Hood *et al*., 1996).

From the symptom observations, it is hypothesised that wounds on lateral roots are the primary point of entry for *P. acaciicola*. The pathogen appears to move through the lateral root's xylem vessels within the stele to the heartwood in the taproot. The narrow corridor of rot joining the heartwood to the vascular cambium is probably the easiest route for *P. acaciicola* to follow and subsequently surround the sapwood. Although the hyphae do not seem to block the xylem vessels, phyllode symptoms indicates a definite shortage of water supply. This could be due to the death of a large proportion of wood (xylem) that would subsequently block the water supply either in the crown or in the lateral roots. Other mechanisms, like the production of toxins, might also be involved in the colonization of the living plant tissue by the pathogen, although it is unknown if *P. acaciicola* produces toxins.

Although *P. acaciicola* was detected in six diseased indigenous plant species, pathogenicity can only be confirmed after the completion of Koch's postulates. The disease incidence (0.9%) of indigenous woody plants within the fynbos and strandveld vegetation is comparatively lower than that of *A. cyclops*. If *P. acaciicola* was responsible for the death of the 10 indigenous plants (0.4%) in which it was detected, the threat that *A. cyclops* poses to these sensitive plant communities overshadows the relatively low risk of *P. acaciicola* being weakly pathogenic to some of the indigenous plant species. Alternatively, it is possible that *P. acaciicola* acts as a non-lethal endophyte or saprophyte that became an opportunistic pathogen on *A. cyclops*. Since all 10 indigenous plants in which *P. acaciicola* was detected were already dead, the latter possibility is favored. The exact trigger that caused the switch to a pathogen on *A. cyclops* is uncertain, but the evolution of increased competitive ability hypothesis (Blossey and Nötzold, 1995), is the most likely explanation.

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2.7 Tables and figures

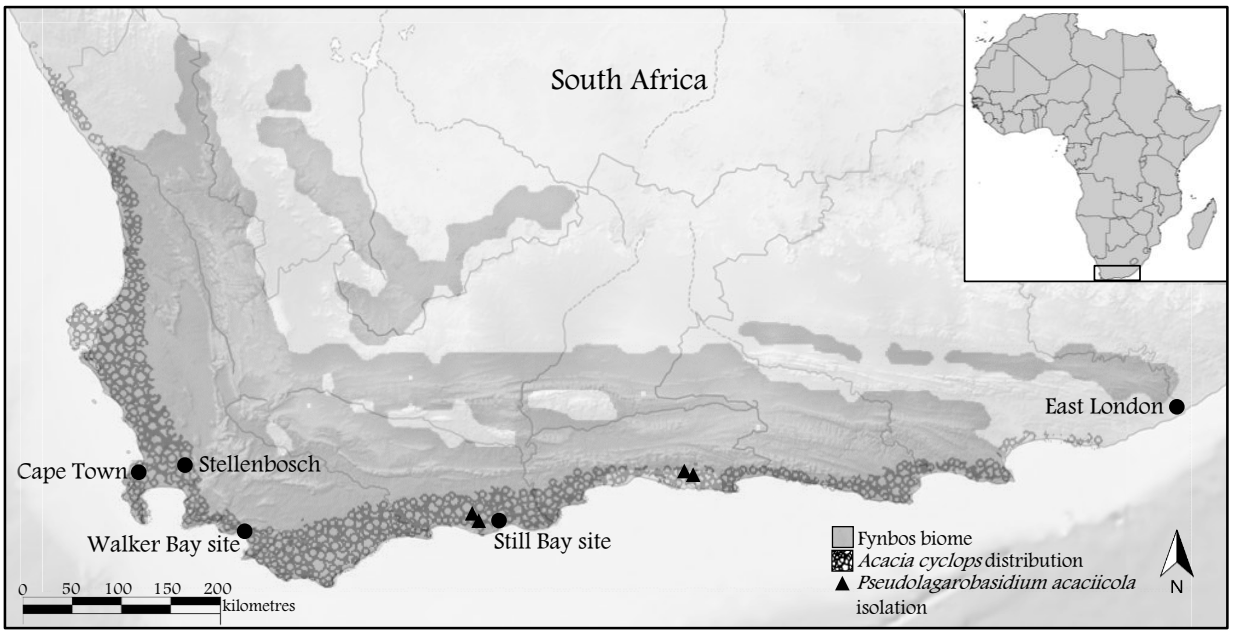

Figure 1. A map of South Africa indicating the locations within the distribution range of *Acacia cyclops* where *Pseudolagarobasidium acaciicola* has been isolated (Wood and Ginns, 2006) as well as the two field sites within the fynbos biome.

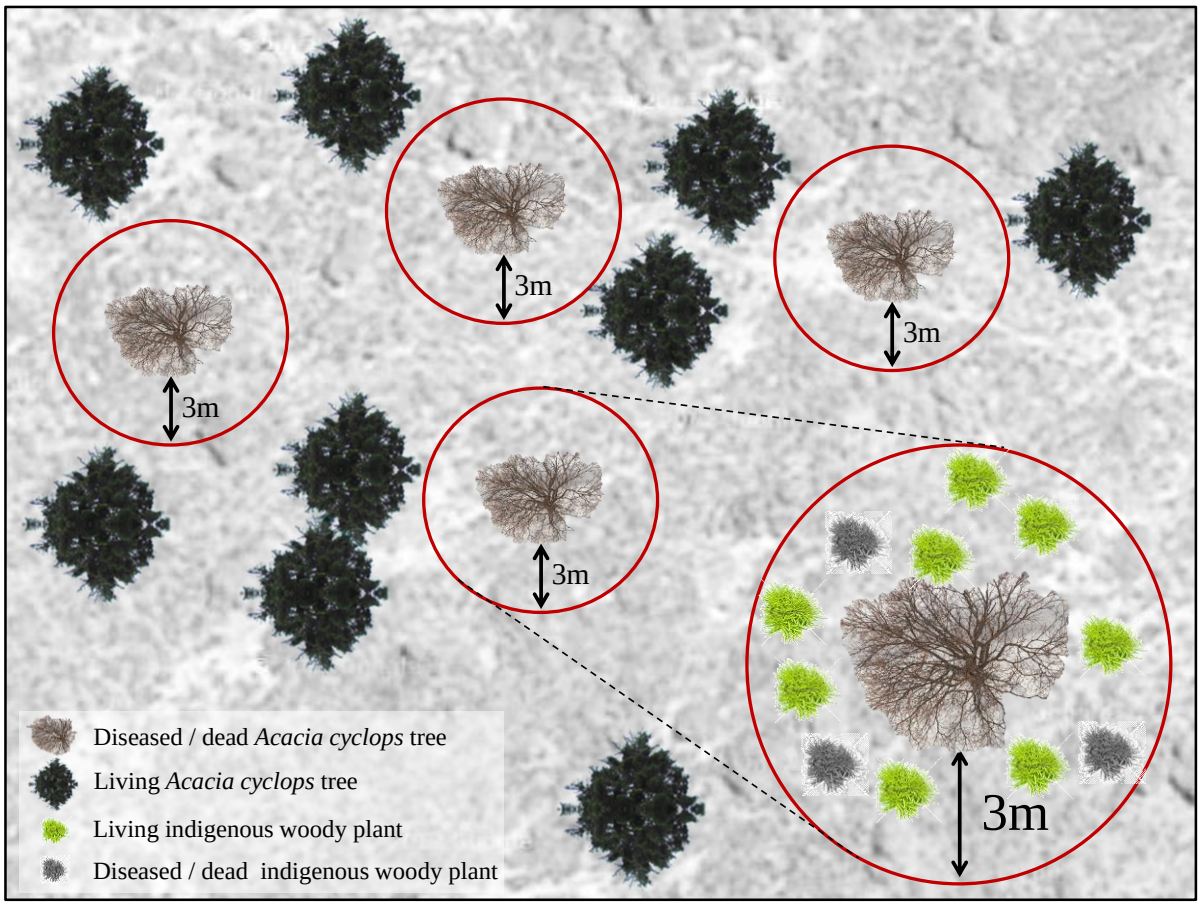

Figure 2. Schematic illustration of a study site indicating the dead or diseased *Acacia cyclops* trees (between live *A. cyclops* trees) around which all indigenous woody plants in a circle with a 3 m radius were recorded, photographed and subsequently identified. Root tissue samples of the identified dead or diseased indigenous plants were taken for later analysis.

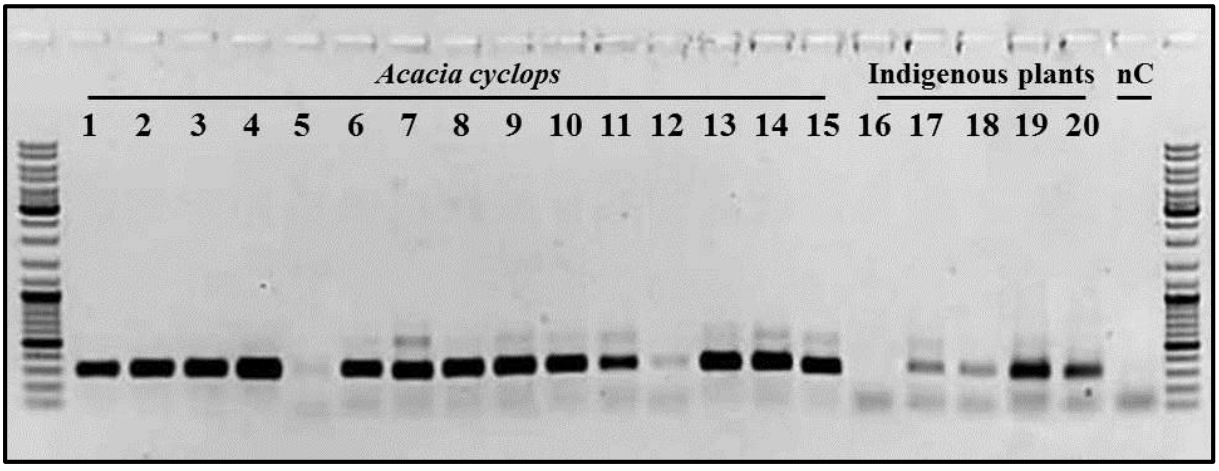

Figure 3. Detection of *Pseudolagarobasidium acaciicola* from the roots of (1-15) diseased *Acacia cyclops* and (16-20)–indigenous plants. The size of digested products was estimated by using a 100bp DNA ladder. PCR was conducted with primers Pac1 and Pac2; nC, negative control.

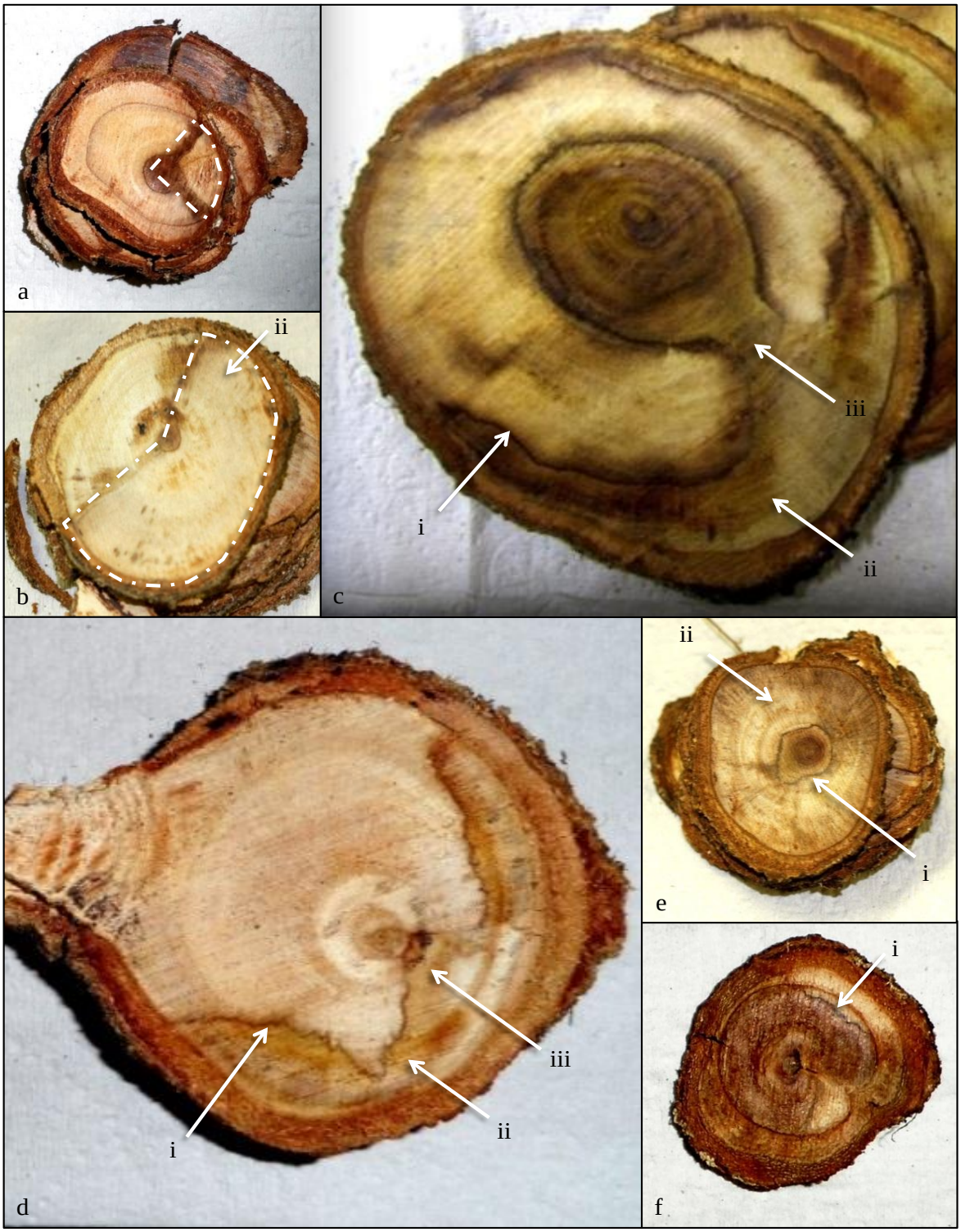

Figure 4. Symptoms observed on sections of *Acacia cyclops* in which *Pseudolagarobasidium acaciicola* was molecularly detected. Sections displayed are of the taproot just above the last lateral root. Symptoms of root colonization range from (a and b) sectorial to (c and d) partial to (e and f) near complete with (i) thin black lines at the disease interface, (ii) a watery appearance of dead wood, and (iii) rot corridors joining the heartwood to the vascular cambium.

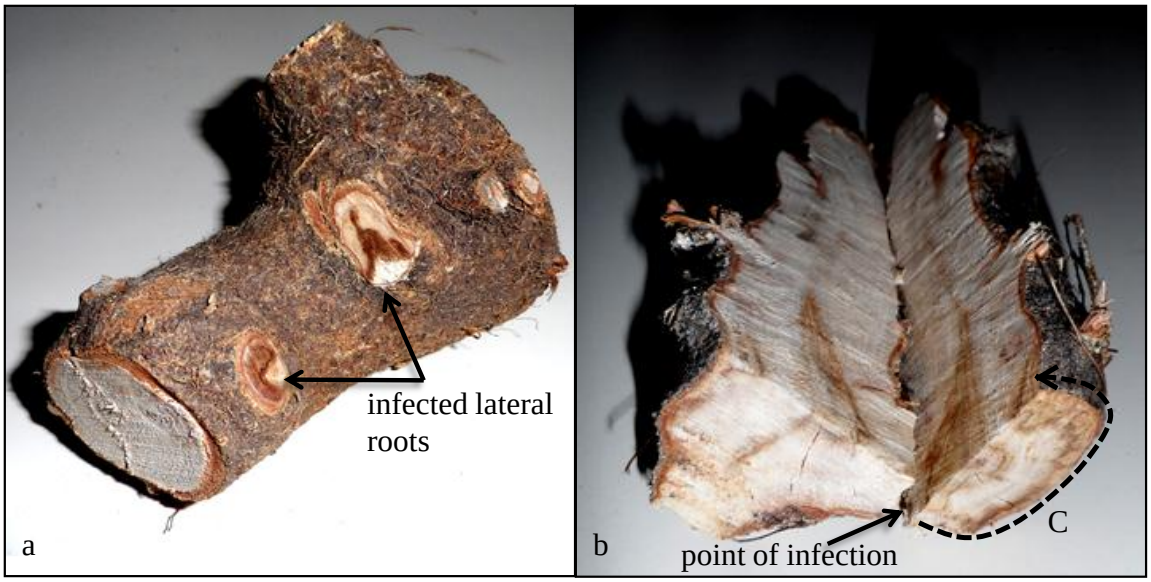

Figure 5. A section made through roots of *Acacia cyclops* from the field in which *Pseudolagarobasidium acaciicola* was molecularly detected. Infection of the taproot can take place via one or more lateral roots (a). The vertical cross section indicates how the pathogen girdles the stem (C) from the point of infection by moving through the vascular tissue (b).

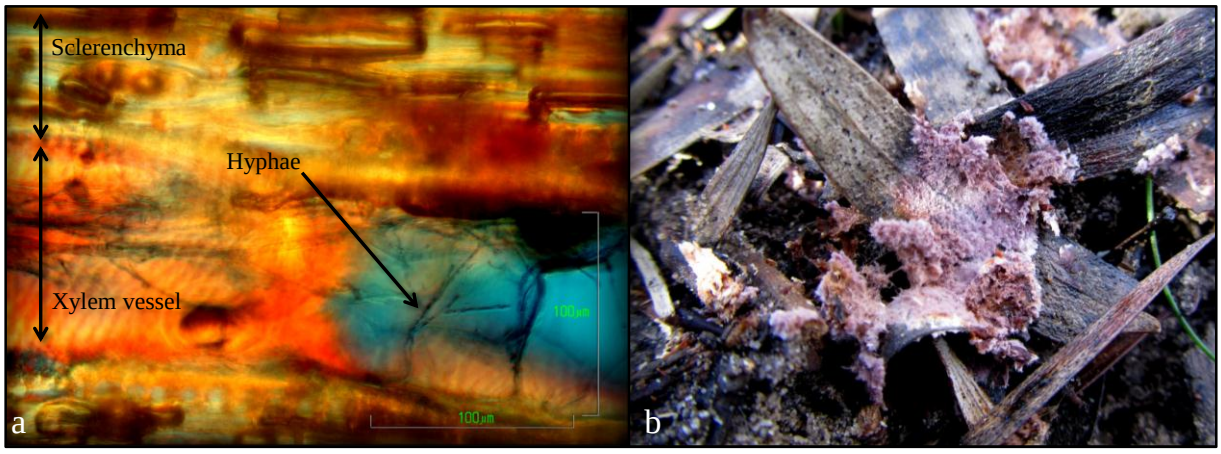

Figure 6. A longitudinal section of a vascular bundle showing the presence of *Pseudolagarobasidium acaciicola* hyphae within a xylem vessel of an inoculated *Acacia cyclops* tree (a) and purple basidiomes of the same pathogen on phyllode litter in the field against the stem of an inoculated *A. cyclops* tree (b).

Table 1. A list of indigenous woody plant species surveyed within a 3 m radius of 100 dead or dying *Acacia cyclops* trees in Still Bay and Walker Bay.

Species	**Family**	**Common name**	**Total**
Acmadenia heterophylla	Rutaceae	Longstem Pinkfanbuchu	4
Acmadenia obtusata	Rutaceae	Dune buchu	12
Agathosma apiculata	Rutaceae	Garlic buchu	2
Agathosma muirii	Rutaceae	Still Bay buchu	4
Aloe arborescence	Asphodelaceae	Krantz aloe	25
Aspalathus quinquefolia ssp. *hispida*	Fabaceae		50
Aspalathus sanguinea ssp. *sanguinea*	Fabaceae	Limestone peabush	59
Asparagus rubicundus	Asparagaceae	Wag-'n-bietjie	113
Berkheya coriaceae	Asteraceae	Wild thistle	5
Carissa bispinosa	Apocynaceae	Num-num	10
Carpobrotus edulis	Mesembryantheaceae	Sour fig	25
Chironia baccifera	Gentianaceae	Christmas berry	19
Chironia tetragona	Gentianacea	Sticky Centaury	59
Chrysanthemoides monilifera	Asteraceae	Tick Berry	180
Chrysocoma tenuifolia	Asteraceae	Bitterbush	67
Cliffortia alata	Rosaceae	Pypsteelbos	102
Cliffortia obcordata	Rosaceae	Diamond Caperose	54
Cliffortia stricta	Rosaceae	Stipuled Caperose	14
Coleonema calycinum	Zygophyllaceae	Confetti bush	15
Conicosia pugioniformis ssp. *muirii*	Mesembryantheaceae	Pigroot	49
Conyza scabrida	Asteraceae	Oven bush	28
Crassula fascicularis	Crasulaceae	Ruiksissie	18
Diosma awilana	Rutaceae		2
Diosma echinulata	Rutaceae	Bitterboegoe	38
Diosma hirsuta	Rutaceae	Rooiboegoe	19
Diospyros dichrophylla	Ebenaceae	Common star-apple	2
Eriocephalus africanus var. *africanus*	Asteraceae	Wild Rosemary	54
Euchaetis meridionalis	Rutaceae		41
Euclea racemosa	Ebenaceae	Dune guarrie	17
Gnidia setosa	Thymeleaceae		2
Gnidia squarrosa	Thymelaeaceae		60
Gymnosporia heterophylla	Celastraceae	Common spikethorn	12
Gymnosporia polyacantha	Celastraceae	Kraal spike thorn	7
Helichrysum crispum	Asteraceae	Hottentot's bedding	135
Helichrysum teretifolium	Asteraceae	Heath-leaf Strawflower	4
Indigofera hamulosa	Fabaceae	Indigo	6
Lauridia tetragona	Celastraceae	Climbing-Saffron	18
Leucadendron coniferum	Proteaceae	Cone bush	6
Leucadendron salignum	Proteaceae	Geelbos	7
Leucospermum praecox	Proteaceae	Still Bay pincusion	1
Lycium ferocissimum	Solanaceae	African boxthorn	3
Metalasia densa	Asteraceae	Blombos	5
Metalasia luteola	Asteraceae	Yellow blombos	4
Metalasia muricata	Asteraceae	White bristle bush	10

Table 1 continued

Species	Family	Common name	Total
Muraltia ericaefolia	Polygalaceae	Tortoiseberry	3
Muraltia spinosa	Polygalaceae	Tortoiseberry	7
Myrsine africana	Myrsinaceae	Cape myrtle	93
Oedera imbricata	Asteraceae	Butterfly bush	2
Oedera squarrosa	Asteraceae	Vierkantperdekaroo	5
Oedera steyniae	Asteraceae	Kalkrandperdekaroo	6
Olea europaea ssp. *africana*	Oleaceae	Wild olive	99
Olea exasperata	Oleaceae	Dune olive	7
Osyris compressa	Santalaceae	Cape Sumach	15
Otholobium candicans	Fabaceae		2
Otholobium fruticans	Fabaceae	Cape Town pea	1
Passerina corymbosa	Thymelaeaceae	Gonna bush	217
Passerina galpinii	Thymelaeaceae	Dune strong bark bush	20
Phylica axillaris	Rhamnaceae	Hard leaf	6
Phylica ericoides var. *muirii*	Rhamnaceae	Heath phylica	18
Phylica stenopetala	Rhamnaceae		6
Polygala myrtifolia	Polygalaceae	September bush	51
Protea obtusifolia	Proteaceae	Limestone sugarbush	3
Protea repens	Proteaceae	Sugarbush	1
Pterocelastrus tricuspidatus	Celastraceae	Cherry wood	10
Pteronia uncinata	Asteraceae	Strandgombos	2
Roepera calcicola	Zygophyllaceae		24
Roepera flexiosum	Zygophyllaceae	Spekbos	16
Salvia africana-lutea	Lamiaceae	Beach salvia	1
Scolopia mundii	Salicaceae	Red pear	2
Searsia crenata	Anacardiaceae	Dune crowberry	29
Searsia glauca	Anacardiaceae	Kuni-bush	158
Searsia laevigata	Anacardiaceae	Dune currant bush	78
Searsia longispina	Anacardiaceae	Spiny currant-rhus	8
Searsia lucida	Anacardiaceae	Glossy current bush	6
Senecio burchellii	Asteraceae	Molteno disease plant	3
Senecio halimifolius	Asteraceae	Tabakbos	9
Senecio umbellatus	Asteraceae	Grounsel	23
Sideroxylon inerme	Sapotaceae	White milkwood	21
Solanum africanum	Solanaceae	Dune nightshade	5
Stoebe muirii	Asteraceae	Krulblaarslagbos	5
Stoebe nervigera	Asteraceae	Steekblaarslangbos	3
Stoebe plumosa	Asterceae	Slangbos	30
Struthiola argenteum	Thymelaeaceae	Featherhead	26
Ursinia anthemoides	Asteraceae	Bergmagriet	2
Wahlenbergia calcarea	Campanulaceae	African bluebell	2
85 Species	27 Families		2432[†]

[†]Individual plants

Table 2. A list of diseased indigenous woody species from a field survey (compared to *Acacia cyclops*) that were tested for the presence of *Pseudolagarobasidium acaciicola* in the roots.

Species	**Family**	**Detected*/ plants†**	**Total surveyed**
Acacia cyclops	Fabaceae	47/100	100
Chrysanthemoides monilifera	Asteraceae	0/2	180
Searsia glauca	Anacardiaceae	0/2	158
Olea exasperata	Oleaceae	0/1	7
Diosma echinulata	Rutaceae	0/1	38
Agathosma apiculata	Rutaceae	0/1	2
Passerina corymbosa	Thymeliaceae	4/7	217
Euchaetis meridionalis	Thymeliaceae	1/1	41
Muraltia spinosa	Polygalaceae	2/2	7
Polygala myrtifolia	Polygalaceae	1/1	51
Leucadendron salignum	Proteaceae	1/1	10
Euclea racemosa	Ebenaceae	1/3	16
11 Species	8 Families	10/22	727

*Number of confirmed *P. acaciicola* identifications after gel electrophorosis and sequence analysis

†Number of dead or dying plant individuals from the specified species of which a root sample was sequenced

Chapter 3

Pathogenicity studies to determine susceptibility of indigenous fynbos and strandveld plants to *Pseudolagarobasidium acaciicola*

3.1 Abtract

Acacia cyclops is a major Australian invasive weed in the species rich strandveld and fynbos vegetation types of South Africa. Observations of *Acacia cyclops* dieback over the past 30 years have lead to the isolation of the causative agent, *Pseudolagarobasidium acaciicola*. This locally occurring fungus is proposed as a mycoherbicide on *A. cyclops*. In order to determine the potential risks associated with using *P. acaciicola* as a mycoherbicide in these vegetation types, pathogenicity trials on indigenous plant species were conducted to give an indication of host susceptibility. A total of 30 indigenous plant species were wound inoculated at two field sites, and potted plants representing 17 indigenous plant species were wound and soil inoculated in a nursery. The optimum growth temperature for *P. acaciicola* was determined in order to understand it's seasonal and landscape preference. Mortality was recorded in five of nine indigenous Fabaceae species, while a single plant each of four other non-Fabaceae species died after inoculation. Only *A. cyclops* seedlings died following soil inoculation. Longitudinal cross sections of stem inoculated plants revealed no systemic infection in Fabaceae species that survived inoculation. Infection in susceptible Fabaceae species was generally far greater than infection in susceptible non-Fabaceae species. The optimum growth rate for *P. acaciicola* was determined at 35°C, indicating an adaptation to summer conditions. Indigenous Fabaceae species display greater susceptibility than species from other families, indicating some level of specificity, although susceptible species can not be phylogenetically circumscribed. Aside from being a facultative pathogen on *A. cyclops*, results from this study and previous work suggest that *P. acaciicola* is primarily a saprophyte and an occasional opportunistic pathogen on some indigenous Fabaceae, possibly only being a weak opportunistic pathogen on some non-Fabaceae species. However, the risk of not effectively managing *A. cyclops* populations in these threatened vegetation types outweighs the risk associated with using *P. acaciicola* as a mycoherbicide. The use of *P. acaciicola* as a mycoherbicide on *A. cyclops* would be

recommended, given that sufficient monitoring is undertaken in the areas where this mycoherbicide is applied. This mycoherbicide should not be viewed as an independent solution to the *A. cyclops* problem in South Africa, but should be incorporated into an integrated management strategy to control this weed effectively.

3.2 Introduction

Acacia cyclops A. Cunn. ex G. Don. (Fabaceae, Mimosoideae) was originally introduced from Australia to South Africa to stabilize the shifting sand dunes along the southern coast. This weed has since successfully established and spread throughout the limestone fynbos and strandveld vegetation along the coast of of South Africa. Dense stands of *A. cyclops* are a major threat to these habitats that are exceptionally rich in biodiversity (Manning, 2007). Partial success have been achieved by the introduction of two classical biological control agents on *A.cyclops* from its natural distribution range that either feed on the seed or cause galling around the flower heads (Impson *et al*., 2011). The current effect that the biological control agents have on *A. cyclops* is however not sufficiently controlling these populations (le Roux *et al*., 2011) The introduction of a biological control agent that attacks the vegetative parts of the tree have not been considered as a result of the weed's commercial value as firewood in an otherwise tree-poor environment (van Wilgen *et al*., 2011).

A natural dieback of *A. cyclops* has been observed across a large part of its distribution range from as early as the 1960's (Taylor, 1969). The causal organism, *Pseudolagarobasidium acaciicola* (Polyporales, Basidiomycota) Ginns was isolated from the roots of diseased trees (Wood and Ginns, 2006). This locally occurring Basidiomycete, currently under consideration for use as a mycoherbicide, could offer a compromise between the conservation of the indigenous biota and the commercial value of this tree to poor local communities. Before commercialization of this mycoherbicide, it is important to perform a risk assessment in order to determine the effect of amplified populations of *P. acaciicola* on the indigenous flora. Earlier work (Chapter 2) found a low incidence of dead and dying indigenous woody plants (DDIPs) (0.9%) in the immediate area around dead or dying *A. cyclops* trees. *Pseudolagarobasidium acaciicola* was detected in approximately half of the sampled *A. cyclops* and the DDIPs. The probability of *P. acaciicola* acting as a saprophyte on a variety of indigenous plant species was well supported.

When doing research to determine the long term effect of a pathogen on its surrounding environment, multifaceted methodologies including comparative testing of pathogens in

natural- and controlled environments are necessary (Flory and Clay, 2013). The susceptibility of *A. cyclops* and indigenous plant species to *P. acaciicola* should therefore not only be determined in glasshouses, but also in the field where these plants occur naturally.

The "centrifugal phylogenetic method" (CPM) has been used widely as a guideline for host-specificity testing for potential weed biocontrol agents (Wapsphere, 1974). This concept of testing is based on the principle that host susceptibility is directly associated with phylogenic relatedness. The ultimate aim of the concept is to determine whether susceptible plants in a test group can be circumscribed within a monophyletic clade or not. The test group focuses on species occurring within the proposed introduction area. Wapsphere stresses the importance of identifying the closest phylogenetically related species to the target species within the environment of its proposed introduction prior to pathogenicity trials.

Of the 28 fungal biological control pathogens released internationally and reviewed by Barton (2012), 107 plant species were found to be susceptible in glasshouse pathogenicity trials prior to release. However, only six of these species were actually found to be affected in the field post-release. This implies that glasshouse pathogenicity trials are overly sensitive when assessing risk prediction of the true post-release effects of fungi in the field. Therefore glasshouse trials should only provide an indication of pathogenicity rather than mirror a prediction of what could happen in the field. It should be kept in mind though that this review only discussed biotrophic fungi used as biological control agents and that the situation might be different for necrotrophic biocontrol fungi.

The large majority of pathogens target selected genotypes, species, genera or families, displaying different degrees of specificity (Keen and Staskawicz, 1988; Gilbert and Webb, 2007). It is important to establish where *P. acaciicola* fits in this continuum of host specificity. If this pathogen is not species-specific on *Acacia cyclops*, it could be genus specific (infecting most of the *Acacia* species), family specific (infecting most Fabaceae) or non-specific (infecting a wide range of species). Susceptibility could be influenced by morphologic host similarities (Puchalska *et al*., 2006). Although initial pathogenicity trials suggest that *P. acaciicola* might be non-specific, these were artificial inoculations on thin stemmed plants under glasshouse conditions (Wood, 2001). Although a non-specific pathogen may infect multiple plant species, resistance and disease tolerance may vary between these plants that act as hosts for the pathogen, leading to more serious symptoms on some infected plants when compared to others (Holah and Alexander, 1999; Dobson, 2004). This suggests that *P. acaciicola* could cause symptoms other than dieback on other hosts even though it kills *A. cyclops*.

Although the factors influencing the susceptibility of plant species to fungal pathogens are poorly understood, plant species with a close phylogenetic relationship to susceptible species are more likely to be susceptible than a distantly related species (Gilbert and Webb, 2007). Since *P. acaciicola* is more likely to infect plant species that are phylogenetically closely related to *A. cyclops* than non-related species, it is important to determine the closest related species to *A. cyclops* within its invaded distribution range in South Africa. The tribe Acacieae has been proven not to be monophyletic and subsequently the genus *Acacia* is now split into five genera namely *Acacia*, *Acaciella*, *Mariousousa*, *Senegalia* and *Vachellia* (Brown *et al*., 2008; Miller *et al*., 2011). All Australian species are in *Acacia*, including *A. cyclops*, while species indigenous to Africa are now divided between the genera *Vachellia* and *Senegalia* (Miller *et al*., 2013). *Acacia cyclops* therefore has no genus-level indigenous relatives that could be threatened by *P. acaciicola* within its distribution. The risk assessment consequently reverts back to the broader Fabaceae group that includes a large number of species.

Manning (2007) lists 668 species of Fabaceae in the fynbos biome alone, including species like *Xiphotheca phylicoides* A. L. Schutte and B.–E. van Wyk, which is critically endangered (Raimondo *et al*., 2009). Of the three small populations of this species surviving in the Outeniqua Mountains, two are already under threat by the invasive weeds *Hakea gibbosa* (Sm.) Cav. and *Pinus patula* Scheide & Deppe. However their distribution is ecologically distinct to the coastal plains invaded by *A. cyclops* and where dieback has been long observed to occur (Taylor, 1969).

Temperature has a major impact on the growth and consequent interaction intensity between fungi and their hosts (Traill *et al*., 2010). Temperature also plays an essential role in a fungus' persistence in the environment (Boivin *et al*., 2006). No optimum growth temperature for *P. acaciicola* has been determined. Determining the optimum growth temperature for *P. acaciicola* will give a better understanding of the pathogen in terms of its favoured conditions and adaptation to a specific environment. It is hypothesized that the changing climate could be the cause of emerging diseases caused by pathogens and their vectors sensitive to changes in temperature, rainfall or other environmental conditions (Patz *et al*., 2005).

This study aims to give an indication of the host specificity of *P. acaciicola* through pathogenicity trials in the field and nursery. The pathogenicity trials will include plant species in which *P. acaciicola* was detected (Chapter 2) in order to complete Koch's postulates to confirm whether *P. acaciicola* caused the death of these plants. Furthermore, the results of

the pathogenicity trials should give a clearer indication of the biological character of *P. acaciicola*, i.e. endophytic, saprophytic or pathogenic. A phylogenetic analysis of the susceptible species will attempt to circumscribe these species. Ultimately, the risk that *P. acaciicola* poses to the indigenous flora if applied as a mycoherbicide is to be determined. In addition, by determining the optimal growth temperature for *P. acaciicola*, it's seasonal and landscape preferences should be better understood.

3.3 Materials and methods

3.3.1 Inoculum preparation

A *P. acaciicola* isolate (PPRI 7335, DAOM 230979, CBS 115543) from the Plant Protection Research Institute's (PPRI) National Collection of Fungi was selected for the inoculation trials based on its proven pathogenicity on *A. cyclops* (Wood and Ginns, 2006). Sorghum seed on which *P. acaciicola* was grown was used as inoculum. The seed was soaked overnight, dried and autoclaved before adding potato dextrose agar (PDA) blocks (± 3 mm^3) on which *P. acaciicola* had grown for 7 days. The fungus was incubated on the sorghum seed for 7 days at 35°C. Controls were inoculated with autoclaved sorghum seed.

3.3.2 Field inoculations

Indigenous woody plant species, including Fabaceae species, were inoculated at two strandveld sites in the Western Cape. The plants were inoculated in the Walker Bay- (near Stanford) and Still Bay area. The age of the plants ranged between approximately 6 months and 6 years old. Plants were inoculated by cutting through the bark and cork cambium with a blade, and placing one to five treated sorghum seed, depending on the size of the plant, directly into the cavity. The wound was sealed with parafilm. For each species at a site, two plants were inoculated and one used as a negative control. *Acacia cyclops* plants were also inoculated to serve as positive controls. In total, 33 different species were inoculated in the field, 21 species at Walker Bay (Table 1) and 23 species at Still Bay (Table 2). Species were selected based on relatedness to *A. cyclops*, abundance, availability and stem diameter. Plants were monitored every 2 months for 14 months (Walker Bay) and 9 months (Still Bay) respectively.

3.3.3 Nursery inoculations

In an experiment with indigenous woody plant species in pots under shade netting at the Plant Protection Research Institute (PPRI) near Stellenbosch, five plants per species were stem inoculated as above, five were soil inoculated and five untreated as negative controls (see Table 3 for list of inoculated species). For soil inoculations, 1 gram of colonized sorghum was placed in each of two 5–cm deep holes in the soil near the stem of the plants. The stem diameters at the point of inoculation and height of all plants were recorded at the time of inoculation. Plants were left for 8 months after inoculation and watered 3 days per week. The height of every plant was measured after 8 months. Two series of *A. cyclops* controls, both young and old plants, were inoculated in the nursery.

3.3.4 Isolation from inoculated plants

Longitudinal cross sections were made with a thin circular saw of all inoculated plants at the end of the study, or subsequent to a plant's death. The nature of the infection, if any, was visually analysed and photographed. Isolations were made from these longitudinal transects by plating out small wood blocks (± 3 mm^3) from approximately 10 mm above and 10 mm below the point of inoculation onto potato dextrose agar amended with streptomycin (PDA^+) in Petri dishes. Pure cultures were made of fungal growth resembling that of *P. acaciicola*. Its unique characteristics allowed for the distinction of *P. acaciicola* from other fungi based on the colour, growth rate and stereo microscope photos of the isolated cultures. These cultures were compared to pure cultures of the *P. acaciicola* strain that was used as inoculum to confirm identification.

3.3.5 Phylogenetic analysis of Fabaceae species

The available ITS sequences of Fabaceae species which were inoculated, as well as non-Fabaceae species which seemed susceptible, were obtained from GenBank. Inoculated Fabaceae species from Wood (2001) were also included. A sequence alignment program, MAFFT version 6 (Katoh and Toh, 2008), was used to align these sequences and adjusted manually in Sequence Alignment Editor v. 2.0a11 (Rambaut, 2002). To perform the maximum parsimony analysis, Phylogenetic Analysis Using Parsimony (PAUP* v. 4.0b10) was used. The heuristic search option was used to conduct the analysis with 10 random taxon

additions. For the branch swapping algorithm, tree bisection and reconstruction (TBR) was used with the option of saving a maximum of 10 trees with a score equal to or greater than 5 (Harrison and Langdale, 2006). All alignment gaps were treated as missing data. Characters all had the same weight and were not ordered. The calculations of bootstrap support values were based on 100 heuristic search replicates and 10 random taxon additions. Retention index (RI), tree length (TL), consistency index (CI) and rescaled consistency index (RC) values were calculated for parsimony.

3.3.6 Optimum temperature for growth of *P. acaciicola*

The growth experiment was carried out by using the strain of *P. acaciicola* used for preceding inoculations (PPRI 7335, DAOM 230979, CBS 115543). The optimum growth temperature of *P. acaciicola* was determined by using 9–cm–diameter non–vented plastic Petri dishes containing 20 ml PDA^{+}. A sterile cork borer was used to cut 5–mm–diameter plugs from the margin of actively growing *P. acaciicola* colonies on PDA^{+}. The plugs were placed in the centre of the Petri dishes and incubated at 15°C, 20°C, 25°C, 30°C, 35°C and 40°C respectively. Ten replicate dishes were placed at each temperature and incubated under dark conditions. All plates were sealed with parafilm and kept in a paper bag. Increase in colony size was measured at two diameters at 90° to each other every 24 hours for 4 days. The experiment was repeated twice. The radial growth rate ($mm.day^{-1}$) on each Petri dish was determined by calculating the growth difference between the 24 hour intervals. An appropriate analysis of variance (ANOVA) was used to analyse the data from the growth rate trial as influenced by temperature. In order to demarcate the optimal temperature regime for fungal growth, Fisher's least significant difference (LSD) was calculated to identify significant differences between temperature effects at a confidence interval of 95%. Addinsoft XLSTAT Version 2013.4.05 (www.xlstat.com) was used for all statistical analysis.

3.4 Results

3.4.1 Field pathogenicity trials

3.4.1.1 Walker Bay

Plants from two indigenous plant species died subsequent to their inoculation (Table 1). Both plants of the Fabaceae species *Indigofera brachystachya* (DC.) E.Mey. and *Otholobium bracteolatum* (Eckl. & Zeyh.) C.H.Stirt. died after 2 and 6 months respectively. All inoculated *A. cyclops* died within 6 months after inoculation. *Pseudolagarobasidium acaciicola* was successfully re-isolated from the roots of all the dead plants. The other inoculated indigenous Fabaceae species, namely *Psoralea pinnata* L. and *Aspalathus calcerea* R. Dahlgren, survived the inoculation. Eighteen other plant species and their controls survived (Table 1).

3.4.1.2 Still Bay

Nine months after inoculation, two of the four inoculated *A. cyclops* plants had died, while the remaining two experienced wilt symptoms. Both controls survived. The only indigenous plant species that died was *O. bracteolatum* (after 2 months), a species belonging to the Fabaceae family. All controls and the other 22 inoculated plant species, including the legume, *Aspalathus sanguinea* Thunb. subsp. *sanguinea*, survived the inoculation (Table 2).

3.4.2 Nursery pathogenicity trials

After 2 weeks and 1 month respectively, all the stem– and soil–inoculated young *A. cyclops* had died. All five of the mature stem inoculated *A.cyclops* were dead after 9 months. Mortality was recorded in three indigenous Fabaceae species. All stem inoculated *Podalyria calyptrata* (Rctz.) Willd. and *Virgilia divaricata* Adamson were dead 2 months after inoculation. Two stem inoculated *Psoralea pinnata* plants died 6 and 9 months after inoculation respectively. Outside of the Fabaceae family, one stem inoculated *Metalasia muricata* (L.) D. Don and one *C. monilifera* (L.) T. Norl. plant (both Asteraceae) died after 2 weeks, while one stem inoculated *Searsia lucida* (L.) F.A. Barkley (Anacardiaceae) and one stem inoculated *Olea exasperata* Jacq. plant (Oleaceae) died after 7 months (Table 3).

Pseudolagarobasidium acaciicola was successfully re-isolated from the stems and roots of all the dead plants, except *M. muricata*. Leaf senescence was recorded on the stem inoculated *Olea europaea* L. subsp. *africana* (Mill.) P.S. Green plants after 9 months, although none have died. All other inoculated and control plants were alive after 9 months.

3.4.3 Growth, infection and re-isolation

Nursery–inoculated *Sideroxylon inerme* L. was the only plant species where a clear distinction in growth between stem–inoculated and control plants could be observed (Figure 1). The average growth of the stem–inoculated individuals of *S. inerme* over 8 months was 70 mm, compared to the 334 mm recorded in controls and 246 mm in soil inoculated plants. Additionally, leaf senescence was observed in all stem–inoculated *Olea europaea* L. subsp. *africana* (Mill.) P.S. Green in the nursery, although none of these plants died. The longitudinal cross sections of all the dead plants, but also living plants revealed systemic infections (Figure 2). Infections did however seem more restricted in the dead non-Fabaceae species than Fabaceae species. Both of the inoculated *A. cyclops* plants at Still Bay still living displayed serious infections. Systemic infections outside the Fabaceae family include two Asteraceae, two Anacardiaceae, two Oleaceae and one Thymeleacea species (Table 4). *Pseudolagarobasidium acaciicola* was successfully re-isolated from all dead plants, with the exception of the two Asteraceae plants in the nursery. Re-isolations of *P. acaciicola* were successful from as high as 200 mm above and 100 mm below the point of inoculation in some cases.

3.4.4 Phylogenetic analysis

Phylogenetic analysis revealed no significant phylogenetic relatedness between susceptible species within the Fabaceae family. Subclades from the parsimony tree correlated well with the respective tribes within the Fabaceae (Figure 3).

3.4.5 Optimum temperature for growth

The linear growth rate of *P. acaciicola* with regard to temperature is illustrated in Figure 4. The maximum mean growth rate of *P. acaciicola* was recorded at 35°C (18.2 $mm.day^{-1}$), while the slowest mean growth was recorded at 40°C (0.3 $mm.day^{-1}$). Fisher's

LSD test revealed a significant difference between the mean radial growth rate of all temperature treatments ($p < 0.05$), except when comparing 30°C to 35°C ($p = 0.176$) and 15°C to 40°C ($p = 0.488$). The growth rate increased as the temperature increased from 15°C to 35°C.

3.5 Discussion

When considering mortality found in the field pathogenicity studies, not only *A. cyclops*, but certain Fabaceae species, seem to be susceptible. This confirms that *P. acaciicola* should not be classified as a species–specific pathogen on *A. cyclops*, but supports the idea that *P. acaciicola* might act as an opportunistic pathogen on indigenous Faboideae within its distribution range (Hallenberg *et al.*, 2008). Although two of the inoculated *A. cyclops* plants survived at Still Bay, wilt symptoms and advanced systemic infections suggests that these plants would not have survived for much longer. Since the inoculated *A. cyclops* at Still Bay are approximately the same size than those at Walker Bay and all died within a relatively short period of time, other abiotic or biotic factors might explain the lower mortality rate at Still Bay. Climatic–related stress is most probably not the cause, as the sites experience very similar weather patterns. The most noticeable difference between these inoculation sites is the soil type. Rooikrans inoculated at Walker Bay are rooted in very sandy soils (closely associated with strandveld), while those at Still Bay are rooted in shallow limestone soils (closely associated with limestone fynbos) (Mucina and Rutherford, 2006). Keeping in mind that plants were stem inoculated, soil type could not be the direct cause of mortality difference. Soil type could however affect the hardiness and subsequent resistance of a tree, or drought stress experienced by plants. The same phenomenon was observed in a previous study where *A. cyclops* was inoculated with *P. acaciicola* at strandveld and limestone fynbos sites respectively (Wood, pers. comm.).

Stem diameter does not seem to play a major role in susceptibility between species, although it clearly influences the resistance of plants within a species to inoculation. This can be illustrated by the thicker stemmed (16.2 and 25.2 mm) *O. bracteolatum* at Walker Bay taking three times as long to die off when compared to the thinner stemmed (6.1 and 7.8 mm) *O. bracteolatum* plants at Still Bay. One might argue that mature plants with stems greater than 60 mm (like *P. pinnata*) could take longer than 14 months to show signs of susceptibility. For exactly this reason, younger individuals of the same species were subsequently inoculated in the nursery and cross sections of each plant would also reveal

whether *P. acaciicola* caused a systemic infection, even if the plant is still alive. Mature trees in the field were selected, firstly, due to a lack of younger trees at the sites, and secondly to give an indication of whether *P. acaciicola* has any short term effects on mature indigenous species. Interestingly, the Fabaceae species with the smallest stem diameter, *Aspalathus calcarea* R. Dahlgren, survived the inoculation at Walker Bay. At the same time *A. sanguinea* subsp. *sanguinea*, a vulnerable endemic severely threatened by alien acacias (Raimondo *et al*., 2009), survived the inoculation at Still Bay. This provides evidence that susceptibility to *P. acaciicola* most likely does not encompass the whole of the Fabaceae family, but rather a selection of species within this family, and that the classification of a plant plays a more important role in susceptibility than stem diameter.

Although the soil inoculations in the nursery did not result in mortality after 9 months, apart from the young *A. cyclops* seedlings, it could lead to mortality of older plants in the longer term. Wounds appear essential for infection, and nursery plants do not get the same exposure to wound causing organisms as plants in the field. This confirms that stem inoculations would be a more effective method of applying *P. acccicola* as a mycoherbicide than soil inoculations and that *P. acaciicola* is not an aggressively spreading pathogen.

Both *P. calyptrata* and *V. divaricata* seem to be as susceptible to inoculation of *P. acaiicola* as *A. cyclops*, with 100% mortality in stem inoculated plants after 1 month. Wood (2001) recorded 100% mortality for *V. oroboides* in his pathogenicity trials. Although only two *P. pinnata* plants died, systemic infections observed in the living plants suggest that the remainder of the species might have died in due time. These are all fast-growing pioneer species (Coates Palgrave, 2002). High mortality rates have been recorded for fast–growing pioneer species (Dalling and Denslow, 1998) and this relationship can be interpreted as a trade-off to promote growth while chemical defenses are sacrificed in some cases (García-Guzman and Espinosa-García, 2011). One could argue therefore that *P. acaciicola* might target the fast-growing species within the Fabaceae family. However the counter argument would be that *A. cyclops* is a slow–growing species (Coates Palgrave, 2002) and that *Crotalaria capensis* Jacq., which is a fast–growing Fabaceae species (Johnson *et al*., 2002), survived the stem–inoculations. This species did however react differently during pathogenicity trials by Wood (2001), where all three of the inoculated plants died, although these plants were significantly younger and thinner stemmed (Wood, pers. comm.). Even though a large number of isolations have been made from diseased *Virgilia* trees throughout the southwestern Cape, *P. acaciicola* was not identified from any of these isolations (Machingambi, 2013). This indicates that, even though *Virgilia* plants might be susceptible to

P. acaciicola, there is no evidence proving that *P. acaciicola* is a threat to these species in the field.

Eleven of the plant species inoculated in the nursery trials were inoculated in the field at Walker Bay or Still Bay, or both of these field sites (Table 4). All 11 species, with the exception of *A. cyclops*, survived inoculations in the field. Mortality was recorded in three of the 11 species in the nursery. Re-isolations were made from *M. muricata* (after 2 weeks), *P. pinnata* (after 6 months) and *S. lucida* (after 7 months), of which the latter two yielded *P. acaciicola*. *Metalasia muricata* could have died as a result of the large wound made in its thin stem (4.8 mm). The maturity and larger stem diameter of *P. pinnata* plants in the field probably prevented this Fabaceae species from experiencing the same fate as some of the nursery plants. None of the susceptible species in the nursery, that were inoculated in the field, proved to be susceptible in the field, with only a single *S. lucida* and *Olea exasperata* plant displaying infection when sections were made from the stems. This lack of susceptibility in the field could be ascribed to the hardiness and slower growth of plants in the field compared to the nursery. Therefore findings of this study, in support of Barton (2012), indicates that glasshouse pathogenicity trials are not always an accurate reflection of what will happen in the field.

The stem diameter of the mature *A. cyclops* in the nursery resembled those in the field, although the nursery trees were significantly taller. This phenomenon of plants growing slower in the field than the nursery is very common due to increased stress in the field. Regarding mature *A. cyclops*, *P. acaciicola* has a very similar efficacy in the field compared to the nursery. Long-term studies involving the monitoring of mature *A. cyclops* inoculations resulted in 95–100% mortality in the field (Impson *et al*., 2011), although this may take much longer than 12 months (Wood, pers. comm). Another interesting comparison was between the two inoculated *Indigofera* species. At Walker Bay, both *I. brachystachya* plants died 2 months after inoculation, while none of the stem inoculated *I. jucunda* showed any signs of stress after 6 months in the nursery. Even more surprisingly, the stem diameter of *I. brachystachya* was almost five times that of *I. jucunda* on average. Substantial differences in resistance to *P. acaciicola* seem to not only differ between genera, but also within genera in the Fabaceae family.

The observation of systemic infections and re-isolation of *P. acaciicola* were generally in accord with the observed mortalities, although it provided valuable insight into the susceptibility of species like *Searsia crenata* (Thunb.) Moffet and *Olea europaea* subsp. *africana*, in which no mortality was recorded. The fact that none of the isolations from

asymptomatic stems yielded *P. acaciicola* strongly suggests that this fungus does not act as an endophyte as recorded for other closely related fungi (Crozier *et al*., 2006; Chokpaiboon *et al*., 2010). Even though stunted growth was recorded for *S. inerme* in the nursery, no systemic infections were observed. The stunted growth was possibly a result of resource allocation of the plants towards defense mechanisms. Wood (2001) recorded no mortality in *Sideroxylon inerme* L. subsp. *inerme* in a preliminary pathogenicity trial using *P. acaciicola*.

Other plant species inoculated in the present study were similar to some of the plants inoculated by Wood (2001). Although both *A. calcarea* and *A. sanguinea* subsp. *sanguinea* survived inoculations at Walker Bay and Still Bay, respectively, three *A. linearis* (Burm. f.) Dahlg. seedlings inoculated by Wood (2001) resulted in 100% mortality. Wood (2001) recorded 100% mortality in *Olea europaea* subsp. *africana*, which is in accord with the signs of susceptibility of the two *Olea* species in this study. *Chrysanthemoides monilifera* (L.) T. Norl. seedlings survived inoculation by Wood (2001), although a single mortality, three systemic infections and a successful re-isolation of *P. acaciicola* was recorded for the same species in the present study. Inoculation results are not identical to that of Wood, but the same conclusion can ultimately be made with regard to *P. acaciicola*. Since mortality and systemic infections were not only experienced by Fabaceae species, but non–Fabaceae to some extent, the results of the nursery pathogenicity trials supports Wood's (2001) preliminary classification of *P. acaciicola*, in part, as a weak general facultative pathogen.

The isolation of *P. acaciicola* from the most distant margin of lesions from indigenous plants above the point of inoculation proves that the fungus moved up the stem. However this could simply be a result of a localized canker at the point of inoculation that sealed of any water or nutrients to the top adjacent vascular tissue, which then becomes prone to fungal infection. This colonization of dead vascular tissue also supports the hypothesis of *P. acaciicola* as a possible saprophyte on indigenous plant species (Chapter 2).

Not a single mortality, systemic infection or subsequent re-isolation of *P. acaciicola* was recorded in the five DDIP species in which *P. acaciicola* was detected through sequencing analysis in earlier work (Chapter 2). Koch's postulates could therefore not be completed and, consequently, *P. acaciicola* is rejected as the causal organism for the death of these indigenous plant species. This provides further support for *P. acaciicola* as general saprophyte, consistent with the majority of species within the genus and wider order (Nakasone and Lindner, 2012).

Phylogenetic analysis reveals that susceptible species can not be circumscribed, not even within the Fabaceae family and species within the same genus react differently to

inoculation with *P. acaciicola*. Susceptible species are found in all Fabaceae tribes included in the pathogenicity trials, although each tribe also comprised species that were not susceptible or partially susceptible. Plants that are susceptible to *P. acaciicola* could have some other physiological or ecological similarity determining their susceptibility, as also found by Puchalska *et al*. (2006). Apart from the previously discussed theories, this relationship remains speculative.

The optimal growth temperature of 35°C for *P. acaciicola* is relatively high when compared to twelve wood-rotting Basidiomycete species in a similar study done by Boddy (1983). Boddy examined the radial growth of these species and found that the optimum growth rate for all species occurred between 20 and 30°C, while none grew at 40°C. All species examined by Boddy formed part of the fungal class Agaricomycetes as does *P. acaciicola*, with *Bjerkandera adusta* (Willd.) P. Karst. and *Trametes versicolor* (L.) Lloyd forming part of the same order as *P. acaciicola* (Polyporales). An optimum growth rate of 30°C was recorded for both of these species. The average rate of mycelial growth recorded at optimum temperature of 35°C for *P. acaciicola* (18.2 mm.day^{-1}) is three times higher than the average growth rate at optimum temperature in Boddy's study (6.1 mm.day^{-1}). The only Basidiomycete that Boddy studied with an average growth rate at optimum temperature of more than ten was *B. adusta* (12.1 mm.day^{-1}). A comparatively high growth rate would imply that, given the optimal conditions, *P. acaciicola* should be able to effectively spread through the soil from inoculated trees to infect uninoculated trees if used as a mycoherbicide.

Pseudolagarobasidium acaciicola's growth performance at 35°C indicates a preference to hot summer days for infection. Although climate change is a controversial topic, increasing temperatures could have favoured the gradual spread of *A. cyclops* dieback since the 1960's. According to Kruger and Shongwe (2004), the annual mean maximum temperature for Cape St. Blaize, in the center of *A. cyclops'* distribution range, has increased by more than 1°C from 1960 to 2003. The data revealed a significant increase in hot days (30°C to 35°C) in the southwestern Cape during this period. *Pseudolagarobasidium acaciicola*'s high optimal growth temperature might explain why *A. cyclops* dieback is more prominent in the warmer temperate region of the weed's invasive range.

In conclusion, the pathogenicity study determined that susceptibility to *P. acaciicola* inoculation ranges wider than the Fabaceae species, although Fabaceae species generally proved more susceptible. However, the susceptible species could not be phylogenetically circumscribed. No mortality of indigenous non–Fabaceae species was recorded in the field, suggesting that plants in the field are more resistant than nursery pathogenicity trials reflect.

From five DDIPs in which *P. acaciicola* was detected (Chapter 2), none died after inoculation and no subsequent re-isolation of *P. acaciicola* was successful. Therefore Koch's postulates could not be completed for any of the inoculated DDIPs in which *P. acaciicola* was detected. Since *P. acaciicola* was proved not to be the causal organism of the death of these plants, the fungus is likely to primarily, apart from being a pathogen on *A. cyclops*, act as a saprophyte on a range of plants in the strandveld and limestone fynbos. Considering results of the pathogenicity study, *P. acaciicola* might occasionally, apart from being saprophytic, also act as a weak opportunistic pathogen on some indigenous species. However, an earlier field survey (Chapter 2) found very little actual impact on indigenous plant species in habitats where *A. cyclops* dieback is prevalent. The use of *P. acaciicola* as a mycoherbicide on *A. cyclops* is associated with low to medium risk to the natural vegetation and is recommended for use to form part of an integrated management plant for this weed. Monitoring focusing on the state of indigenous Fabaceae species should follow inoculations.

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3.7 Tables and Figures

Table 1. Field pathogenicity trial results on plant species after stem inoculation with *Pseudolagarobasidium acaciicola* at Walker Bay.

		Stem diameter (mm)		
Plant species	**Family**	**St1**	**St2**	**Control**
*Acacia cyclops** (1)	Fabaceae	34.2	28.3	24.7
*Acacia cyclops** (2)	Fabaceae	14.6	16.5	24.4
Otholobium bracteolatum	Fabaceae	16.2	25.2	30.1
Indigofera brachystachya	Fabaceae	15.3	19.3	15.6
*Acacia saligna**	Fabaceae	8.5	14.8	17.8
Aspalathus calcarea	Fabaceae	9	10.2	11.2
Psoralea pinnata	Fabaceae	49.3	62.2	66.6
Chrysanthemoides monilifera	Asteraceae	51.9	62.5	63.5
Metalasia muricata	Asteraceae	48.6	56.6	24.2
Leucodendron coniferum	Proteaceae	35.4	41.7	26.6
Protea obtusifolia	Proteaceae	34.4	37.2	33.7
Gnidia setosa	Thymelaeaceae	9.9	10.3	12.6
Passerina corymbosa	Thymelaeaceae	11.1	9.5	11.6
Searsia crenata	Anacardiaceae	22.5	34	16.3
Searsia lucida	Anacardiaceae	19.6	21.2	17.9
Olea exasperata	Oleaceae	23	25.6	17.8
Myrsine africana	Myrsinaceae	6.9	7.7	4.5
Osyris compressa	Santalaceae	15.4	18.5	13.9
Euclea racemosa	Ebenaceae	17.4	38.8	31.3
Sideroxylon inerme	Sapotaceae	18.4	19.4	20.4
Euchaetis meridonialis	Rutaceae	19.5	29.3	15.1
Heliophila linearis	Brassicaceae	13.1	14.2	14.4
Senecio halimifolius	Asteraceae	23.1	24.5	20

*invasive species; St = Stem inoculated; Highlighted = dead

Table 2. Field pathogenicity trial results on plant species after stem inoculation with *Pseudolagarobasidium acaciicola* at Still Bay.

		Stem diameter (mm)		
Plant species	**Family**	**St1**	**St2**	**Control**
*Acacia cyclops** (1)	Fabaceae	12.7	13.2	14.8
*Acacia cyclops** (2)	Fabaceae	10	17.9	10.5
Otholobium bracteolatum	Fabaceae	6.1	7.8	9
*Acacia saligna**	Fabaceae	17.6	19.1	19.7
Aspalathus sanguinea	Fabaceae	16.5	18.4	9.7
Chrysanthemoides monilifera	Asteraceae	9	11	10
Metalasia muricata	Asteraceae	18.9	29	19.4
Leucodendron linifolium	Proteaceae	14.9	16.2	16
Leucospermum praecox	Proteaceae	27.7	31	21.3
Gnidia setosa	Thymelaeaceae	13.9	21	18
Passerina corymbosa	Thymelaeaceae	16.1	17.3	27.8
Searsia crenata	Anacardiaceae	19.2	26.2	14.9
Searsia lucida	Anacardiaceae	8.8	12.5	17
Searsia glauca	Anacardiaceae	13	13.3	7
Euclea racemosa	Ebenaceae	12.3	20.5	20.7
Diospyros dichrophylla	Ebenaceae	9.4	15.1	13.3
Agathosma muirii	Rutaceae	16.9	28	35.8
Diosma echinulata	Rutaceae	10.2	16.7	11.2
Olea exasperata	Oleaceae	15.7	24.8	15.8
Myrsine africana	Myrsinaceae	7.3	8.5	7.6
Osyris compressa	Santalaceae	13.5	28.2	10.4
Sideroxylon inerme	Sapotaceae	20.6	28.4	15.1
Polygala myrtifolia	Polygalaceae	13	17.3	8.8
Gymnosporia buxifolia	Celastraceae	17.7	18.1	16.2
Solanum quadrangulare	Solanaceae	14.7	16.9	8.7

*invasive species; St = Stem inoculated; Highlighted = dead

Table 3. Pathogenicity trial results of potted plant species after inoculation with *Pseudolagarobasidium acaciicola* in the nursery.

		Stem diameter (mm)														
Plant species	**Family**	**St1**	**St2**	**St3**	**St4**	**St5**	**So1**	**So2**	**So3**	**So4**	**So5**	**C1**	**C2**	**C3**	**C4**	**C5**
Acacia cyclops (young)*	Fabaceae	4.5	4.8	4.9	5.2	4.5	4.6	4.5	4.3	4.7	3.9	4.4	4.4	4.6	5.1	4.9
Acacia cyclops (older)*	Fabaceae	10.6	11.8	12.2	13.3	13.9	13.6	14.5	11.3	15.2	12.7	11.3	13.3	12.7	14	11
Podalyria calyptrata	Fabaceae	9.6	11.6	10.8	10.8	9.2	12.4	7	12	11.4	9.6	11.4	7.8	8.6	13	9
Virgilia divaricata	Fabaceae	10	9.7	9.9	11.2	12.6	10.9	11.9	12.9	12	11.1	13.9	13.5	11.6	12.5	12.1
*Acacia saligna**	Fabaceae	15.8	17.2	16.3	15.3	17.2	15.7	14.6	15.8	16.7	16.4	16.5	17.5	17.6	18.7	19
Psoralea pinnata	Fabaceae	15.4	11.1	14.5	18.1	14.9	17.9	13.2	15.9	11.5	12.3	12.9	12.3	12.1	11.9	11
Crotalaria capensis	Fabaceae	11.3	9.3	10.9	10.5	7.9	11.5	8.7	12.4	10.6	10	14.3	8.2	16	9.1	9.4
Indigofera jucunda	Fabaceae	3.5	3.8	3.1	4.1	3.8	3.2	4.2	3.6	3.5	3.8	3.1	4.2	3.6	3.4	3.4
Metalasia muricata	Asteraceae	5.9	4.6	6.3	4.8	6.1	6.2	5.9	7.4	4.8	4	5.7	5.7	4.1	4.4	6.3
Chrysanthemoides monilifera	Asteraceae	7.9	8.3	6.7	6.9	8.1	7.7	6.5	7.9	7.4	7.2	7.9	7	6.3	7.7	8.2
Searsia crenata	Anacardiacea	7.5	7.8	8.9	9.4	10.3	7.3	5.9	6.1	5.2	11	9.6	8.1	9.8	7.8	10.1
Searsia lucida	Anacardiacea	14.8	11.7	12.2	12.2	12.7	12.5	11.8	12.6	13.6	11.8	12	15.9	11.7	12.9	13.4
Olea exasperata	Oleaceae	10.7	12	16	9.1	10.7	10	9.8	19.7	12.4	10.8	8.5	12.1	10.7	11.1	9.5
Olea europaea	Oleaceae	8.5	12	8.8	11.6	9	11.1	9	8.5	5.9	9.6	9.9	9.5	6.9	9.4	9.8
Myrsine africana	Myrsinaceae	3.4	4.2	4.2	3.6	4.2	4.8	3.7	4.7	3.6	3.5	3.2	4.3	4.6	4.7	4
Polygala myrtifolia	Polygalaceae	10.2	7.3	12.5	9.1	9.5	10.1	10.2	7.9	10.2	10.1	11.7	7.2	9.2	9.6	7.2
Leucodendron salignum	Proteaceae	8.1	6.3	10.1	3.4	3.9	8.8	10.2	6.6	5.5	7.6	5.9	3.6	4.3	6.1	4.3
Agathosma apiculata	Rutaceae	4.3	5.2	4.3	4.6	4.6	4.2	5.2	4.1	4.4	6.4	4.4	6.1	4.2	4.2	4
Sideroxylon inerme	Sapotaceae	13.9	15.6	12.2	10.2	12	14.7	11.4	11.8	13.2	12.1	12.1	19.2	15.7	13.8	14.1
Passerina rigida	Thymelaeace	6.2	6	5.2	7.3	6.7	8	6.4	8.1	7.9	7.1	5.1	8.6	6.4	6.8	6.6

*invasive species; St =stem inoculated; So = Soil inoculated; C = Control; Highlighted = dead

Table 4. The mortality and re-isolation success of plant species after stem inoculations with *Pseudolagarobasidium acaciicola* at Walker Bay, Still Bay and in the nursery.

Plant species	**Family**	**Nursery (5)**	**W-Bay (2)**	**S-Bay (2)**
Acacia cyclops	Fabaceae	●●●●●	●●●●(4)	●●○○ (4)
Acacia saligna	Fabaceae		-	○○
Podalyria calyptrata	Fabaceae	●●●●●		
Virgilia divaricata	Fabaceae	●●●●●		
Otholobium bracteolatum	Fabaceae		●●	●●
Indigofera brachystachya	Fabaceae		●●	
Psoralea pinnata	Fabaceae	●●○**	-	
Aspalathus calcarea	Fabaceae		-	
Aspalathus sanguinea	Fabaceae			-
Indigodera jucunda	Fabaceae	-		
Crotalaria capensis	Fabaceae	-		
Metalasia muricata	Asteraceae	†*	-	-
Chrysanthemoides monilifera	Asteraceae	†○**	-	-
Searsia crenata	Anacardiaceae	*	-	-
Searsia lucida	Anacardiaceae	●*	-	○
Searsia glauca	Anacardiaceae			-
Myrsine africana	Myrsinaceae	-	-	-
Olea exasperata	Oleaceae	●○*	○	-
Olea europaea	Oleaceae	○○*		
Sideroxylon inerme	Sapotaceae	-	-	-
Leucodendron salignum	Proteaceae	-		
Leucodendron coniferum	Proteaceae		-	
Protea obtusifolia	Proteaceae		-	
Leucodendron linifolium	Proteaceae			-
Leucospermum praecox	Proteaceae			-
Passerina rigida	Thymelaeaceae	**		
Passerina corymbosa	Thymelaeaceae		-	-
Gnidia setosa	Thymelaeaceae		-	-
Agathosma apiculata	Rutaceae	-		
Agathosma muirii	Rutaceae			-
Diosma echinulata	Rutaceae			-
Euchaetis meridonialis	Rutaceae		-	
Euclea racemosa	Ebenaceae		-	-
Diospyros dichrophylla	Ebenaceae			-
Osyris compressa	Santalaceae		-	-
Solanum quadrangulare	Solanaceae			-
Polygala myrtifolia	Polygalaceae	-		-
Heliophila linearis	Brassicaceae		-	
Gymnosporia buxifolia	Celastraceae			-
Senecio halimifolius	Asteraceae		-	

● dead plant, systemic infection, *P. acaciicola* re-isolated; ○ living plant, systemic infection, *P. acaciicola* re-isolated
* systemic infection only; † dead plant, systemic infection; - plants unaffected; (#) number of inoculated plants.

Figure 1. Stunted growth is noticeable after 9 months in *Sideroxylon inerme* plants in the nursery that were wound inoculated at the stem (St) with *Pseudolagarobasidium acaciicola*, compared to control plants (C).

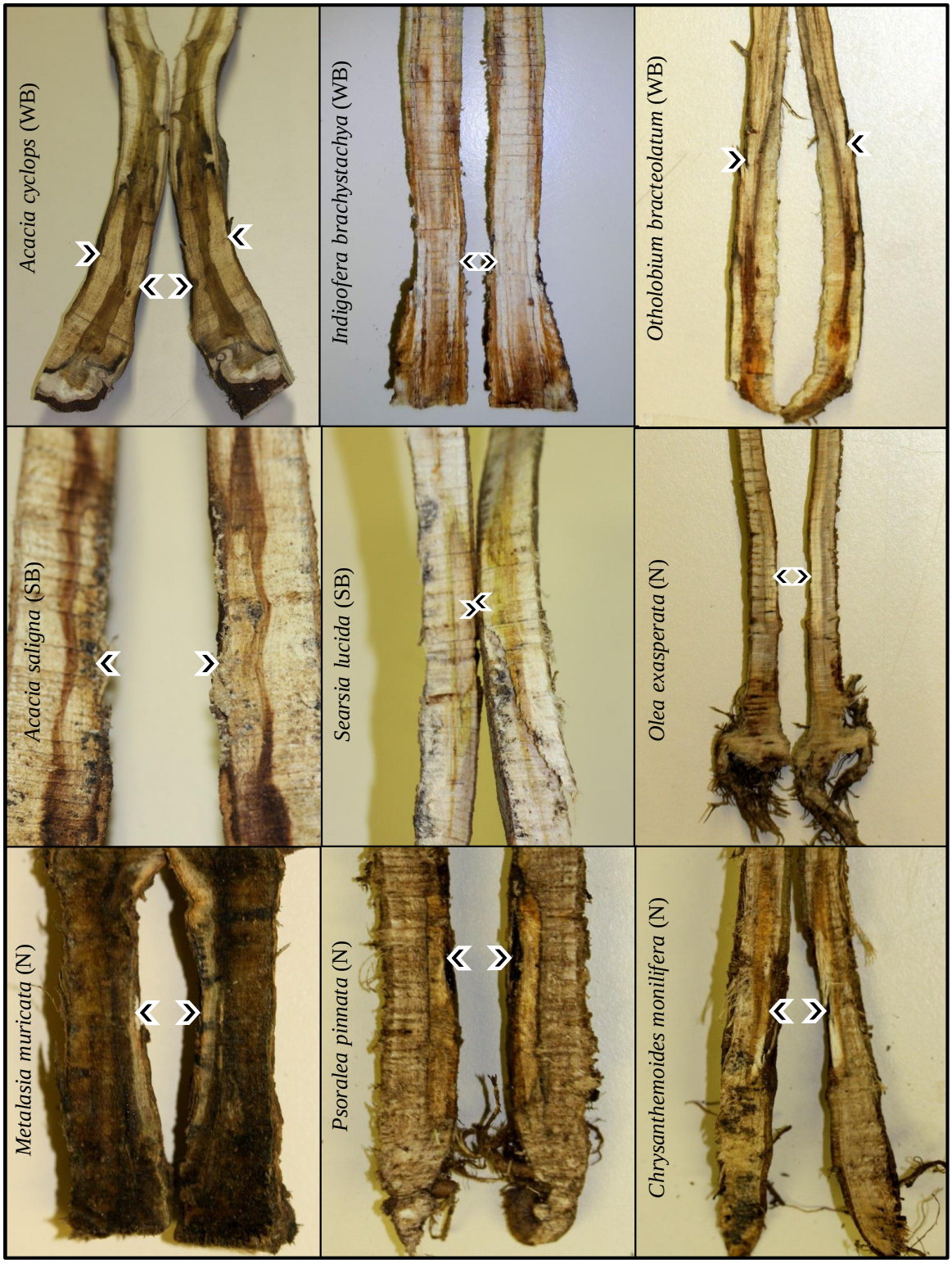

Figure 2. Longitudinal sections of plant species inoculated with *Pseudolagarobasidium acaciicola* at the Still Bay (SB) field site, Walker Bay (WB) field site or the nursery (N) displaying systemic infections. Arrows indicate point of inoculation.

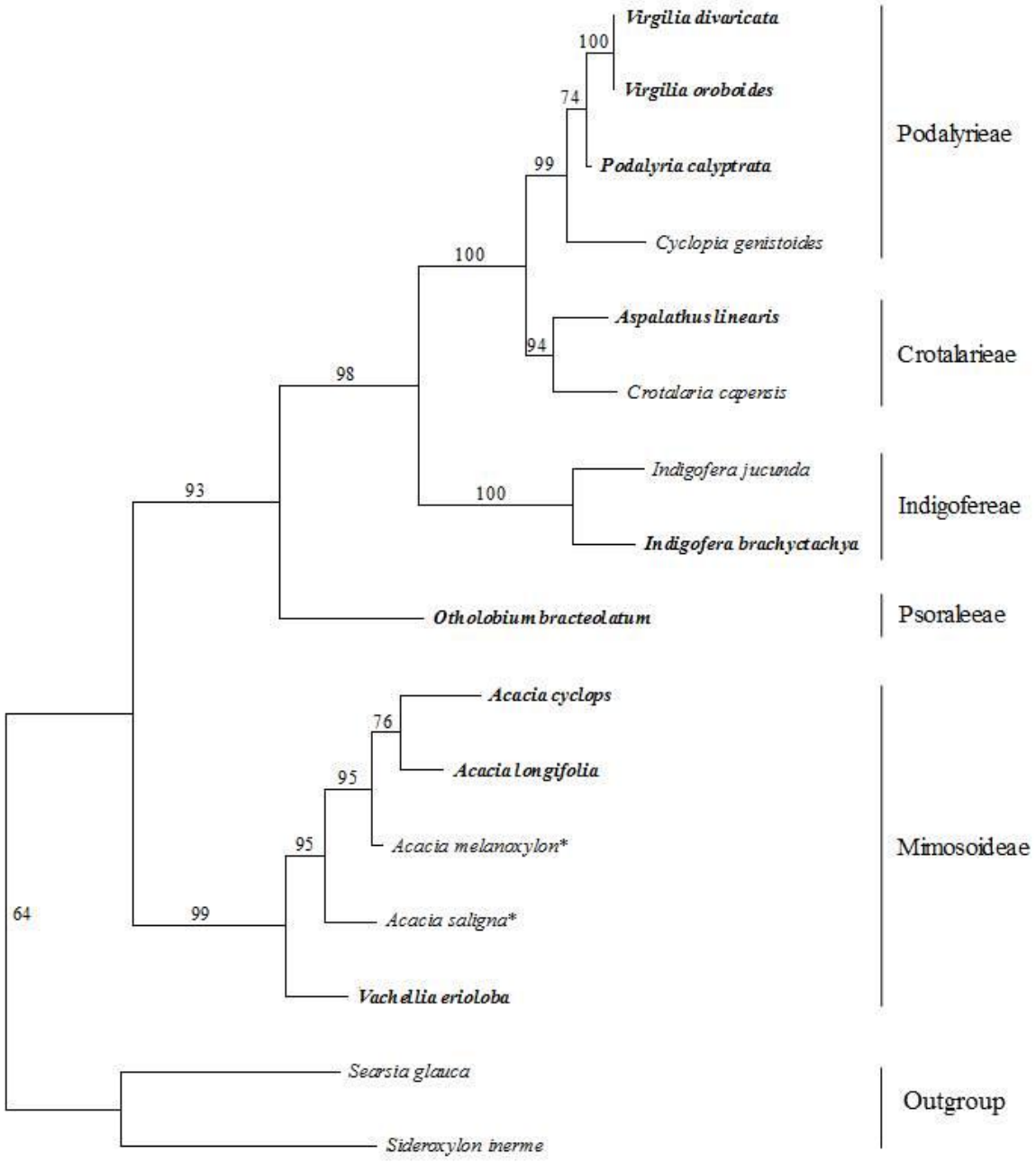

Figure 3. Phylogeny of Fabaceae species based on the ITS1, 5.8S and ITS2 regions of ribosomal RNA. Numbers within the tree represent the bootstrap values. Species that are partially susceptible to *Pseudolagarobasidium acaciicola* are indicated with an asterisk and totally susceptible species are in bold. TL = 4891 ; CI = 0.437 ; RI = 0.313 ; RC = 0.137 ; HI = 0.563.

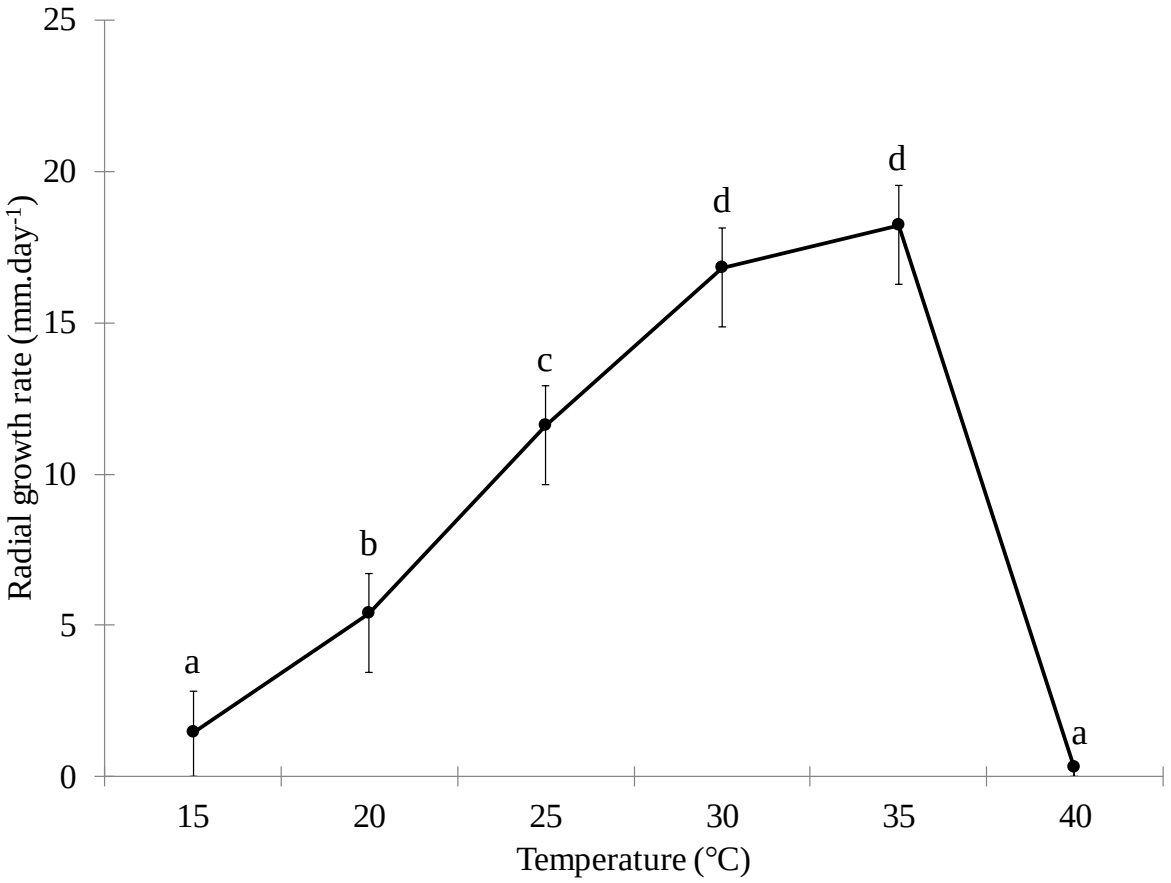

Figure 4: The mean radial growth rate with standard error of *Pseudolagarobasidium acaciicola* measured every 24 hours over 5 days at different temperatures. Means that are significantly different according to Fischer's LSD test are indicated by different letters.

Chapter 4
Concluding discussion

A risk assessment prior to commercialization of a mycoherbicide is essential to foresee possible negative biological interactions. The risk that *Pseudolagarobasidium acaciicola* poses to the indigenous plant species within the threatened limestone fynbos and strandveld vegetation types is investigated in the current study. These results not only provide an indication of the possible effects of using *P. acaciicola* to control *Acacia cyclops* in the field, but also explores the biology and host specificity of this pathogen.

The field analysis of mortality among indigenous woody plants, where *A. cyclops* dieback is prevalent, revealed a very low rate of 0.9% of individual plants in comparison with a substantially higher observed mortality rate of *A. cyclops* at the same sites. There was no phylogenetic relatedness between the dead and dying indigenous plants (DDIPs) and *A. cyclops*. Molecularly, *P. acaciicola* was detected in less than half of the DDIPs and only 0.4% of 2432 indigenous plants that were diseased or dead can be associated with *P. acaciicola*. The presence of *P. acaciicola* within these DDIPs does not imply that *P. acaciicola* is responsible for the state of these plants. Nine of the 11 species represented by the DDIPs formed part of the pathogenicity study to confirm Koch's postulates, although none of these plants showed signs of susceptibility after being inoculated with *P. acaciicola*. It is likely that *P. acaciicola* naturally evolved with the indigenous species as a non-lethal endophyte or saprophyte, becoming an opportunistic pathogen on *A. cyclops* after its establishment as a widespread invasive weed along the southern coast of South Africa. The widespread incidence of the *A. cyclops* dieback could be explained by this association of *P. acaciicola* with indigenous plant species, since these plants act as a source of inoculum. This is the first study to use a field survey in combination with molecular detection to analyse the risk of a potential mycoherbicide.

Pathogenicity trials revealed similar results when comparing field to nursery inoculations, while soil inoculations proved to be ineffective compared to stem inoculations within the given period of time. Four of the nine Fabaceae species proved totally susceptible and one partially susceptible. Two other individual plants from outside the Fabaceae family died subsequent to inoculation during the trial period. The pathogenicity results correlate to some extent with the results of Wood (2001), although a broader range of plant species outside of the Fabaceae family were susceptible. The susceptibility of these species were attributed to

the thin stems in combination with the development of a localized canker at the point of inoculation that seals off water and nutrient transport to the rest of the plant. In contrast to localized cankers, systemic infections were observed in *A. cyclops* stems that spread deep into the roots (Wood, 2001).

Another hurdle to be cleared before recommending *P. acaciicola* as a mycoherbicide is the commercial value of *A. cyclops* and the consequent dependents of this trade. Programmes developed for the management of alien invasive plants should prioritise target areas while considering the benefits of these plants to local residents (de la Fontaine, 2013). Field observations suggest spread under natural conditions is slow, implying that the mycoherbicide can safely be applied in conservation areas and farms where *A. cyclops* is unwanted, without having a significant effect on populations of *A. cyclops* in areas where they are of commercial importance. Even if, by chance, any significant die-off of indigenous plant species is observed following the application of *P. acaciicola* as a mycoherbicide, inoculations could simply be ceased. This would subsequently lead to *P. acaciicola* populations returning to natural background levels, an occurrence that has been confirmed in both fungi (Scheepmaker and Butt, 2010) and bacteria (Jackson, 2003).

There may also be a very limited market for this mycoherbicide, as experienced internationally with other registered mycoherbicides (Morris *et al*., 1999). This would imply that the widespread application of *P. acaciicola* is highly unlikely and would most probably be used in localized small scale operations. The mycoherbicide will especially be useful in sensitive environments where mechanical and chemical control methods would cause a great amount of damage. Examples of these environments are dune-, wetland- and estuary systems that are unable to withstand soil exposure caused by felling or are in close proximity to water bodies that are prone to chemical contamination. The use of *P. acaciicola* would allow the gradual die-off of individual *A. cyclops* trees within dense stands without causing major soil exposure or chemical side effects in the ecosystem.

Biological control agents feeding on reproductive parts alone would require a very long time before reducing *A. cyclops* population densities (Rouget and Richardson, 2003). Even if these current biological control agents eventually manage to stabilize *A. cyclops* populations, the loss of biodiversity caused in the interim may be irreversible. In a situation where the cost of the damage exceeds that of the benefits of a species, a biological control agent that targets vegetative parts of a weed, like *P. acaciicola*, should seriously be considered (van Wilgen *et al*., 2011).

With long-term control strategies rarely implemented in non-agricultural ecosystems (Evans, 2000), future management strategies should focus on the development and implementation of cheaper, more user-friendly and environmentally compatible products for use in these ecosystems, which ultimately plays a major role in the functioning of agricultural lands (Power, 2010).

The risk that *P. acaciicola* poses to indigenous plant species can not be assessed in isolation to determine whether this fungus should be recommended as a mycoherbicide. Recommendations should rather be based on the comparative risk; a measure that also incorporates the risk that *A. cyclops* poses to the limestone fynbos and strandveld ecosystems if the weed is not effectively controlled. With the overwhelming evidence of the environmental degradation caused by *A. cyclops* (Richardson *et al*., 1989; Higgins *et al*., 1999; van Wilgen *et al*., 2001; Wilson *et al*., 2011) and the results of the risk assessment performed in this study, *P. acaciicola* can be recommended as a mycoherbicide to control *A. cyclops* in the limestone fynbos and strandveld of South Africa. Field monitoring should accompany application to ensure indigenous plant populations are not negatively affected.

Pseudolagarobasidium acaciicola should however not be regarded as the sole solution to the *A. cyclops* problem in South Africa, but rather form part of a strategy where mechanical, chemical and biological control complement each other in a combined effort to reduce the impact of this invader.

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