The development and comparison of quantitative PCR assays and enzyme-linked immunosorbent assays as rapid detection methods for specific foliar endophytes

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ABSTRACT

Certain species of foliar fungal endophytes found in conifer trees produce anti-insect compounds. Seedlings inoculated with these toxigenic endophytes have increased tolerance to the spruce budworm, *Choristoneura fumiferana*. To facilitate the detection of the fungi in tree samples, polyclonal assays were developed for the pyrenophorol-producing endophytes *Lophodermium nitens* CBS 127939, *Lophodermium nitens* CBS 127941 and *Lophodermium cf. piceae* CBS 127942. Limits of quantification were found to be 50 ng of mycelium for each assay. qPCR assays were developed for the rugulosin-producing endophyte *Phialocephala scopiformis* DAOM 229536 and pyrenophorol-producing endophytes *Lophodermium nitens* CBS 127939 and *Lophodermium nitens* CBS 127941 based on the ITS region of fungal ribosomal DNA targeting genetic polymorphisms unique to those strains. Limits of quantification were found to be 100 ng, 10 ng and 10 ng of mycelium/gram of needle, respectively. Detection methods were compared by testing conifer needle samples inoculated with target endophytes with each assay. The qPCR method was found to be more sensitive, detecting the endophytes in 48% of tree samples. The polyclonal assay detected endophytes in 7% of samples.

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ABBREVIATIONS

ANOVA- analyses of variance

BSA- bovine serum albumin

CBB- Coomassie brilliant blue stain

CBS- CBS Fungal Biodiversity Center Institute of the Royal Netherlands Academy of Arts and Sciences, Utrecht

DAOM- Department of Agriculture, Ottawa, Mycology, Ottawa, ON

ddH_2O- deionized distilled water

dNTP- deoxyribonucleotide

ELISA- enzyme-linked immunosorbent assay

FAM- 6-carboxyflourescein

HRPO- horseradish peroxidase

ITS – internal transcribed spacer

IgG – Immunoglobulin G

kDa- kilodalton (1000 Da)

LOD- limit of detection

LOQ- limit of quantification

MEA- malt extract agar

OD- optical density

PCR – polymerase chain reaction

PMSF- phenylmethylsulphonyl fluoride

RpAb- rabbit polyclonal antibody

SDS – sodium dodecyl sulfate

TAMRA- 6-carboxy-tetramethylrhodamine

TAQ- *Thermus aquaticus* DNA polymerase

TBST- 60.5 g Trizma base 87.6 g NaCl , 5.6 g Tween 20 made up to 1L in ddH_2O pH 7.4

TMB- 3,3′,5,5′-Tetramethylbenzidine substrate (Sigma-Aldrich, St. Louis MO)

Tukey's HSD- Tukey's Honestly Significantly Different Test

INTRODUCTION

1.1 The spruce budworm

Choristoneura fumiferana, the eastern spruce budworm, is one of the most damaging native insects to spruce and fir trees in Canada (NRC 2014). During the cyclical outbreaks, tens of millions of trees can be severely defoliated. Outbreaks may last for several years and the cumulative defoliation of trees causes significant levels of mortality and inhibition of growth in mature softwood forests. These outbreaks result in substantial losses for the Canadian forest industry and forest-dependent communities (NRC 2014).

Spruce budworm moths may lay 100-300 eggs over a period of several days from mid-July to early August, preferring to deposit egg masses on the foliage of conifer trees, specifically *Abies balsamea* (balsam fir) and several different *Picea* species (spruce) (Royama 1984). Eggs hatch after 10 days and the first-instar larvae disperse throughout the tree, tree stand or even beyond, being carried by the wind. These larvae spin hibernacula in which they overwinter, emerging in the spring as second-instar larvae and proceed to feed on their host trees. Second-instar larvae prefer 1-2 year old needles and pollen cones, but during the third to sixth instar, the larvae attack new branch shoots (Royama 1984).

It takes many years for a budworm population to grow to a level of noticeable defoliation (Miller 1975). A pre-outbreak density of less than five feeding larvae per tree takes approximately four years to reach the endemic level of 2000 larvae per tree, which causes the defoliation of new

shoots. Population density then grows over the fifth and sixth years to 20,000 larvae per tree, stripping the new and old needles. This causes the tree to decline while the population continues to grow in the eighth and ninth years, killing tree tops. From the tenth to fourteen years dominant trees die and the mortality of a productive stand may reach 80% (Miller 1975).

Budworm populations notoriously fluctuate between extreme levels and at very high densities, and larvae cause extensive damage to fir and spruce stands, even to the point of killing mature trees (Royama 1984). Pre-emptive measures to decrease the severity of budworm outbreaks include spraying chemical insecticides, pre-commercial thinning of susceptible tree species or the use of biological insecticides such as *Bacillus thuringiensis (Btk)* and insect growth regulators (Maclean et al. 2002).

Btk is a biological pesticide that has been registered for use in Canada for over 30 years. It is a gram positive soil bacterium that produces proteinaceous crystalline inclusions during sporulation. This inclusion, a δ-endotoxin, exhibits highly specific insecticidal activity against Lepidoptera (Carisey et al. 2004). Many improvements have been made to the original formula and dose estimation of *Btk* but aerial sprayings still show variable efficacy due to the inherent constraints of the product. *Btk* has to be ingested by the larvae to be effective, and once activated by the alkaline pH and proteolytic activity of insect gut fluids, the toxin perforates the midgut epithelium with leads to feeding cessation and ultimate death by septicemia (Carisey et al. 2004). The *Btk* formulation needs to be applied to foliage using aerial spray methods. The efficacy of *Btk* is also affected by the nutritional and chemical quality of food ingested by the insect. Spruce

budworm larvae fed an artificial diet of medium quality were more vulnerable to *Btk* than larvae on the high quality food (Bauce et al. 2002).

New Brunswick has 2.8 million ha of productive Crown forest, 3.2 million ha of productive industrial and private woodlots and 1.0 million ha of non-productive forest land, over 75% of which contain some spruce and fir species, susceptible to spruce budworm damage. Over 55% of the productive forest in northern and central New Brunswick is considered highly or moderately vulnerable to the spruce budworm (Maclean et al. 2002). The province of Quebec lost 180 million m^3 of timber in the spruce budworm outbreak in the 1970s (Caresy et al. 2004). A major epidemic started in 2012 in eastern Quebec leading to severe defoliation in 2013 (Société de protection des forêts contre les insectes et maladies 2014).

Populations of spruce budworm in New Brunswick have been reported to oscillate over periods of 35 years during the last two centuries (Royama 1984). These oscillations depend on mortality factors during the third to sixth larval instars such as parasitoids, diseases and a complex of unknown causes which were termed 'the fifth agent' by Royama in 1984. There are several hymenopterous and dipterous parasitoids that attack spruce budworm larvae throughout all of its instar stages. These wasps insert their eggs into the body of a budworm larva via an ovipositor and second generation wasps emerge and kill their host in the following summer (Royama 1984).

Major predators include spiders and small insectivorous birds from the family *Parulidae* such as warblers, but these major predators are not thought to contribute to the primary population oscillations during the analysis of severe outbreaks (Royama 1984). Mook (1963) found that birds did not seem to consume budworm larvae before their sixth-instar, finding only 10% of larvae found in gizzards were below the 6^{th} instar. This implies that the birds would have only fed on the budworm for a few weeks each season, making it a small factor in the overall budworm population. The spider species that will consume budworm larvae are web spinners which may kill small dispersing larvae but cannot attack large larvae (Miller 1975).

A thorough examination of weather data and budworm outbreak data by Royama in 1984 concluded that weather appeared to have little effect on the survival of feeding larvae. In 1963, Neilson et al. found bacterial, fungal and viral diseases such as nuclear and cytoplasmic polyhedroses (where the virus reproduces in the nucleus of host cells and forms crystals in host fluids, causing death by decay), to be infrequent.

Royama also postulated, in 1984, that his studies of a fir stand that was heavily infested with spruce budworm showed mortality in feeding larvae to be caused by parasitism and the operation of another 'agent'.

1.2 Endophytes

The term 'endophyte' refers to fungi that live inside of plants and cause no visible symptoms of disease (Bacon & White 2000). Fungal endophytes are diverse, and can be found in a variety of plant hosts such as seaweed (Garbary & London 2002), cool season fescue grasses (Clay 1988), fruit bearing plants (Whitesides & Spotts 1991; Schena et al. 2002) and conifer trees (Carrol & Carrol 1978).

Hyphae of fungal endophytes grow in the apoplasm of the plant which supplies the fungus with nutrients required for growth and secondary metabolite production (Kuldau et al. 2008). Analysis of cool fescue apoplasm consisted of glucose and fructose, substances to support fungal growth (Kuldau et al. 2008). Fungal endophytes in grasses grow as elongate, sparsely branched hyphae through the intercellular spaces parallel to the long axis of leaves and stems of plant tissue (Clay 1990). Fungal endophytes are incorporated into the seeds of grass as hyphae grow into the developing ovules and seeds of infected maternal plants (Clay 1988). The transmission to the next generation is through the seed, i.e. are transmitted vertically. After germination, the fungus and plant grow in a coordinated manner, supporting a viable endophyte for the lifespan of the leaf (Schmid et al. 2000). The rate of endophyte infection increases over time in pasture and forage grasses, and old pastures has a much higher infection rate than younger ones (Clay 1988).

In 1988, Clay proposed five characteristics of a 'mutualistic symbiont' referring to endophytic fungi that have been proven to be true of endophytes in many subsequent studies. Characteristics included that the endophyte was ubiquitous in given hosts and caused minimal disease

symptoms, that transmission was vertical or efficiently horizontal, that the fungus grew throughout host tissues, that it produced secondary metabolites with likely antibiotic or toxic properties and that the endophyte could be associated to a known herbivore or pathogen antagonist.

Mechanisms by which photosynthesis and water relationships might be altered by endophyte infection are associated with a plants hormonal metabolism. Studies of photosynthate metabolism of two different host-endophyte associations have revealed that the fungi rapidly convert plant sucrose into sugar alcohols that plants are unable to metabolize, reducing feedback inhibition of photosynthetic rates and resulting in increased growth of the plant (Clay 1988). *Neotyphodium* endophyte species were shown to alter the root length of host fescue and sequester aluminum in response to mineral stresses such as phosphorus deficiency (Malinowski & Belesky 2000).

1.3 Endophytes in cool season fescue

The most widely studied and understood occurrence of fungal endophytes is that of fescue grass in agro-ecosystems. For over 40 years, significant research has been devoted to identifying and understanding the cause of tall fescue toxicosis; a symptomless endophytic infection of tall fescue grass (*Schedonorus phonenix*) with detrimental effects on grazing livestock (Belesky 2009). The fungal endophyte *Neotyphodium coenophialum* grows intercellularly in stems and leaf sheaths of its host plant and provides a variety of benefits.

Tall fescue became the forage grass of choice in North America because of its ability to withstand severe weather, inhospitable soil and consistent demands of beef operations. These environmental stressors on the plant-fungus association influenced the nutritive value of the grass, including alkaloid production (Belesky & Bacon 2009). Fungi from the Clavicipitaceae family, such as *N. coenophialum*, produce biologically active compounds, mostly alkaloids with pharmacological properties (Belesky & Bacon 2009). Fungal endophyte toxins such as alkaloids vary between host species, plant parts, age, seasonal changes and with environmental stressors. In adult plants of tall fescue grass, alkaloid concentrations are higher in young tissue, coinciding with the endophyte hyphal density (Clay 1990).

Subsequent studies have shown the impaired health and performance of livestock grazing on tall fescue (Hoveland et al. 1980; Stuedemann & Hoveland 1988). Tall fescue toxicity syndrome or 'fescue toxicosis' expresses symptoms such as fescue foot (dry gangrene), fat necrosis and 'summer slump' characterized by unsightly appearance, reduced weight gain and excessive salivation (Belesky & Bacon 2009). Symptoms appear from chronic consumption of the ergot alkaloid compounds produced by specific fungal endophytes. Endocrine imbalances were also commonly observed, causing very low birth weights, fewer live births, less pregnancy and reduced milk production (Fribourg et al. 1991). Symptoms were especially severe in mares (Belesky & Bacon 2009).

A defensive mutualism has been proposed where the endophytic fungi defend their plant host against animal and insect herbivory, defending their own resources as well. Ergot alkaloids produced by endophytes in grasses have been famously studied for their effects on mammals; these compounds have a vasoconstricting effect, constricting the small blood vessels to the brain and organs resulting in dementia, convulsions and gangrene. They also have a typically bitter taste and grazing animals quickly learn to avoid infected grasses. However, the greatest ecological effect that grass endophytes have is their influence on insect herbivores. Studies have shown that larvae fed on infected plants have a lower rate of survival, lower mass gain and a longer larval duration than larvae fed on uninfected plants (Clay et al. 1985; Hardy et al. 1985). Insects exhibit a clear preference for uninfected plants (Barker et al. 1984). This lack of herbivory is what allows for the increased growth of host plants. Ryegrass and tall fescue both showed significantly greater growth in studies comparing greenhouse growth of colonized and un-colonized plants (Clay 1987).

1.4 Endophytes as biological controls

Some endophytes are a significant economic problem in beef and dairy industries because of their detrimental effects on livestock. Others endophytes are an attractive potential agent for reducing pest damage on non-fodder grasses such as those on lawns, golf courses and athletic fields (Potter & Braman 1991; Alumai et al. 2009).

Concerns with the potential adverse environmental impacts of chemical insecticides polluting soil (Harner et al. 2009) and water (Williams et al. 1988; Hallberg 1989) have increased the

attention to possible biological control agents. Exploiting naturally occurring enemies to control pest populations is an attractive way to reduce the use of harmful and unspecific chemicals. Endophytic fungi are specific to target organisms and have little effect on other species and the environment. They are self-replicating, making constant applications unnecessary (Schardl et al. 2004; Miller et al. 2009). However, their use as control agents requires an extensive understanding of the interactions between the plant and insect targets in natural systems and much research has been done exploring these relationships (Clay 1989; Clay & Hola 1999; Clark et al. 1989; Henson 2002; Miller 2011).

The broad biological and economic possibilities of endophytes in fescue led to a wide interest into the research and understanding of these mutualistic plant-fungal associations (Funk et al. 1997). The value of using endophytes in grasses became apparent to turfgrass scientists, integrated pest management researchers and even homeowners. Endophytes from the Neotyphodium genus were found in ryegrass, fine fescues, grasses and sedges throughout the world (Funk et al. 1997). Elite perennial ryegrasses chosen for their stress tolerance and insect resistance were selected without the realization that their attractive qualities were elicited, in part, from their innate endophytes. Once this was discovered, turfgrass breeders adopted these endophytes into their programs. Seed producers began promoting 'endophyte-enhanced' products to turf professionals and the general public.

1.5 Endophytes in conifers

Carrol and Carrol (1978) described the incidence of "internal fungal infections" on a medium scale sampling of North American coniferous hosts and suggested that conifer needle endophytes had been inadequately explored. They also hypothesized the functions for of the wide-spread occurrence of endophytes and suggested possible benefits such as the antagonism towards pathogenic needle parasites or decrease in the palatability for grazing insects, as seen in fescue grasses.

The prevalence and ecology of conifer endophytes have been studied in commercially important conifer species. Foliar endophytes have shown to produce toxins that reduce insect growth (Miller 1986; Sherwood-Pike et al. 1986; Findlay et al. 1995; Findlay et al. 1997; Miller et al. 2008; Sumarah et al. 2008; Sumarah & Miller 2009). Early experiments proved the toxicity of specific endophyte extracts to HeLa cell lines (immortal human cell lines used in scientific research) and the spruce budworm (Miller 1986).

Given the beneficial effects that endophytes have on plant hosts such as grasses, there are several ecologically and commercially important areas where an improved understanding of forest tree endophytes is essential. Endophytes have a significant role in the interaction between trees and insects hosts, such as the case of conifer trees and the spruce budworm. The biotechnological potential of endophytes in agriculture is being explored but progress in the forestry industry is lacking (Pirttila & Frank 2011).

Efforts have been placed on exploring the effects of the foliar endophyte *Phialocephala scopiformis* on the spruce budworm (Miller 2011). The occurrence of *P. scopiformis* and its toxins in needles, specifically the toxin rugulosin, reduced spruce budworm development in growth-chamber studies (Miller et al. 2002). Studies in the nursery (Sumarah et al. 2005) as well as in the field (Sumarah et al. 2008; Miller et al. 2009) have shown the fungus persists in the tree and concentrations of toxins accumulate to levels high enough to have an impact on insect growth (Miller 2011). Miller et al. (2008) studied the impact on larvae placed on branches of trees infected with the rugulosin-producing endophyte. The presence of the fungus and its toxin produced a statistically significant decrease in budworm growth compared to controls and a dose-response growth reduction was seen. This demonstrated that the majority of the effect on larval growth caused by the toxin. The use of foliar endophytes on seedling stock used for reforestation could have potential to be a useful approach to increase the innate resistance of the trees to insect pests.

1.6 Natural products of endophytes

Only a few plants have been studied in relation to their endophytic biology. The opportunity to find new and interesting endophytes is very large and shows potential in many different areas. It is estimated that there are 1.5 million species of fungi (Hawksworth 2001) and more biodiversity breeds more chemical diversity and potential useful natural products. There is a constant general call for new antibiotics, chemotherapeutic agents and agrochemicals that are effective and have minimal environmental impact. Due to safety and environmental issues, many synthetic agricultural agents have been targeted for market removal. This has created the need for new

ways to control farm pests and pathogens. Novel natural products and the organisms that produce them have generated opportunities for drug innovation and agrochemical discovery. Endophytes are seen as a great potential source for bioactive natural products because there are so many of them occupying ecological niches (Schultz et al. 2002; Sumarah & Miller 2009; Aly et al. 2011; Kusari et al. 2012). Antibiotics are low molecular weight compounds produced by microorganisms that show biological activity against other microorganisms and are active at low concentrations (Demain 1981). Many natural products have been isolated from endophytes and have been observed to inhibit phytopathogens, bacteria, other fungi and viruses (Strobel & Daisy 2003). *Crytosporiopsis quercina* is an endophyte associated with hardwood tree species in Europe. It exhibits antifungal activity against important human pathogens and produces several bioactive compounds termed 'cryptocandins' with antifungal activity (Strobel et al. 1999). These compounds are currently being examined as natural chemical control agents for the pathogenic fungus *Magnaporthe grisea* (Strobel & Daisy 2003) which destroys rice crops.

1.7 Metabolism

Primary metabolism occurs in all living organisms and is essential for growth. Metabolism is the life-sustaining chemical reactions that occur inside of living cells, allowing them to grow, reproduce and respond to their environment. Secondary metabolism is closely linked with primary metabolism and occurs when an organism is stressed, e.g. nutrient limitation, chemical irritant. Extrolites have been widely accepted as a defence system for fungi, and are often found to be antifungal, antibacterial or show anti-tumour activity (Keller et al. 2005). Metabolites are often bioactive and associated with specific stages of morphological differentiation. Many

natural products are considered plant secondary metabolites, including morphine and quinine (Keller et al. 2005).

Rugulosin (figure 1) is a secondary metabolite with an intense yellow colour produced by a variety of fungi such as *Penicillium* (Bouhet et al. 1976) and *Phialocephala* (Sumarah et al. 2008). It is an anthraquinone metabolite derived from acetate. Rugulosin shows antibacterial and insecticidal activity (Calhoun et al. 1992; Miller et al. 2002; Sumarah et al. 2008; Miller et al. 2009). It was first reported as a fungal toxin from woody plants in 1992 (Calhoun et al. 1992) isolated from endophytes found in balsam fir.

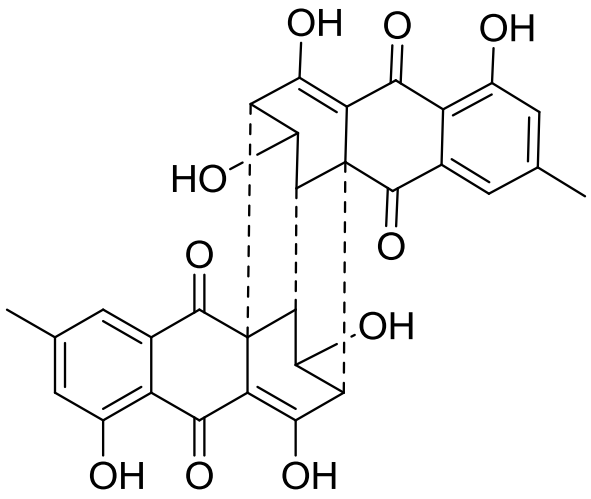

Figure 1. Structure of rugulosin.

The secondary metabolite pyrenophorol (figure 2), has been isolated from *Lophodermium* species (Sumarah et al. 2011), *Byssochlamys nivea, Alternaria alternata, Drechslera avenae* and *Phoma* species (Zhang et al. 2008). It is a polyketide and is bioactive against *Microbotryum violaceum* (Zhang et al. 2008) and *Saccharomyces cerevisae* (Sumarah et al. 2011) in disc diffusion assays. This potential production of antifungal toxins interests researchers because pathogenic fungi such as *Cronartium ribicola* (white pine blister rust) have destroyed the commercial value of western white pine in western Canada (Sumarah et al. 2011). White pine blister rust breaks down the bark of trees and the pathogen spreads until infected the branch or stem is girdled from nutrients and dies.

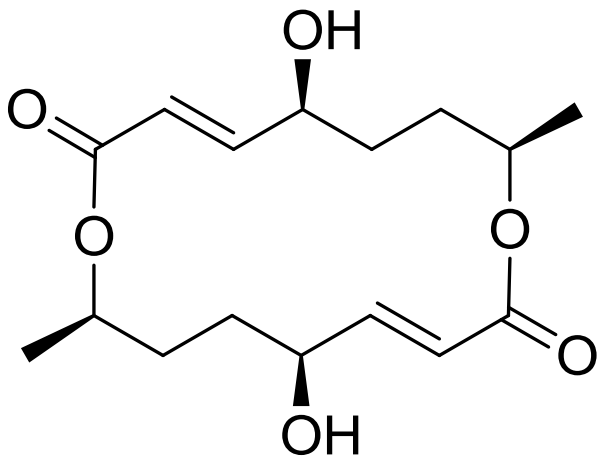

Figure 2. Structure of pyrenophorol.

1.8 Phialocephala and *Lophodermium*

Phialocephala scopiformis (figure 3) was first described by Kowalski and Kehr in 1995, isolated from living branches of conifer trees in Germany. *P. scopiformis* differs from previously described *Phialocephala* species by having elongated and complex conidogenous heads, composed of many series of branches resulting in a 'broom-like' conidiophore (Kowalski & Kehr 1995). The genus *Phialocephala* is primarily isolated from soils, root and decayed wood in northern temperate regions (Addy et al. 2000). *Phialocephala fortinii* is the most common root fungi with a very wide geographic range and no known host specificity (Addy et al. 2000). Species of *Phialocephala* have been cited as being the dominant dark septate endophyte in the aboveground tissues of many temperate tree species (Grunig et al. 2008). Dark septate root endophytes (DSE) are a miscellaneous group of conidial or sterile fungi that infect roots (Jumpponen & Trappe 1998). *Phialocephala piceae, P. glacialis* and *P. scopiformis* have been isolated from living needles of *Picea* species (Grunig et al. 2008). *P. scopiformis* is also a known producer of the anti-insectan compound rugulosin (Sumarah et al. 2008) making it a species of interest for biological control of conifer insect pests.

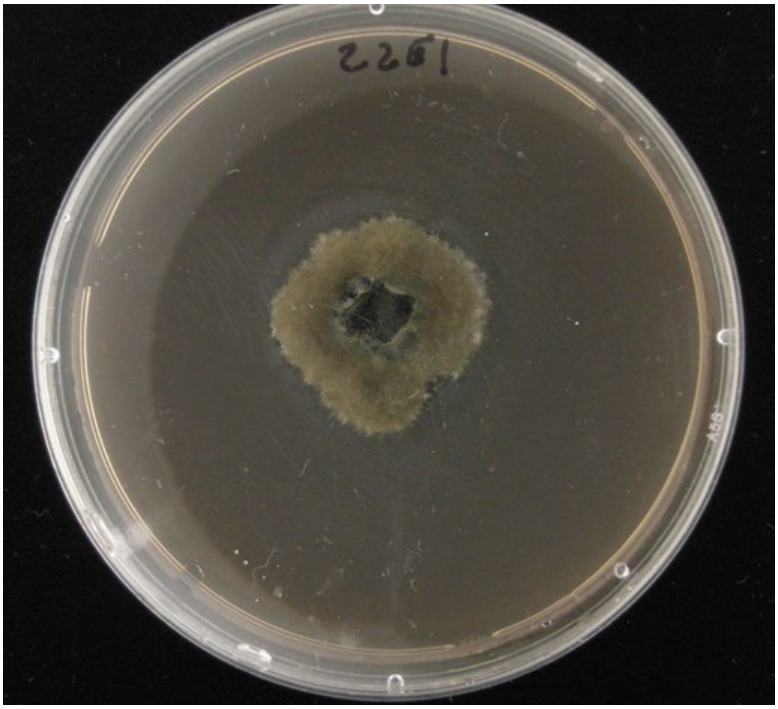

Figure 3. *Phialocephala scopiformis* on 2% MEA plate.

Colony of P. scopiformis on 2% malt extract agar at 3 months old.

The genus *Lophodermium* (figure 4) comprises ascomycetous fungi that are mostly asymptomatic endophytes on a variety of plant hosts (Ortiz-García et al. 2003). It is a large, complex genus, differentiated from other genera in the family Rhytismataceae by their filiform ascocarps that open with a longitudinal slit. They are among the most common species of foliar endophytes isolated from pine and spruce trees (Ortiz-García et al. 2003). There are over 20 *Lophodermium* species known to colonize conifer trees and only one, *L. seditiosum*, is a major pathogen causing needle-cast (Ortiz-García et al. 2003). Species of *Lophodermium* were isolated from superior white pine trees and their fungal extracts contained many different compounds with interesting antifungal bioactivity, including the compound pyrenophorol (Sumarah et al.

2011). Figure 5 represents the phylogenetic relationships of selected pyrenophorol-producing strains of *Lophodermium* used in the presented study.

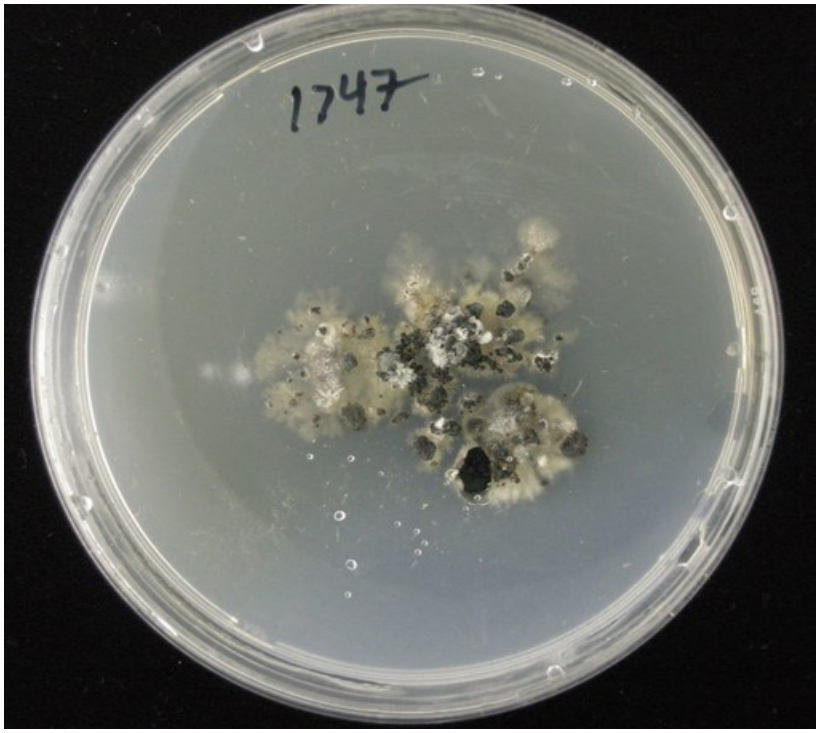

Figure 4. *Lophodermium nitens* on a 2% MEA plate.

Colony of *L. nitens* on 2% malt extract agar at 3 months old.

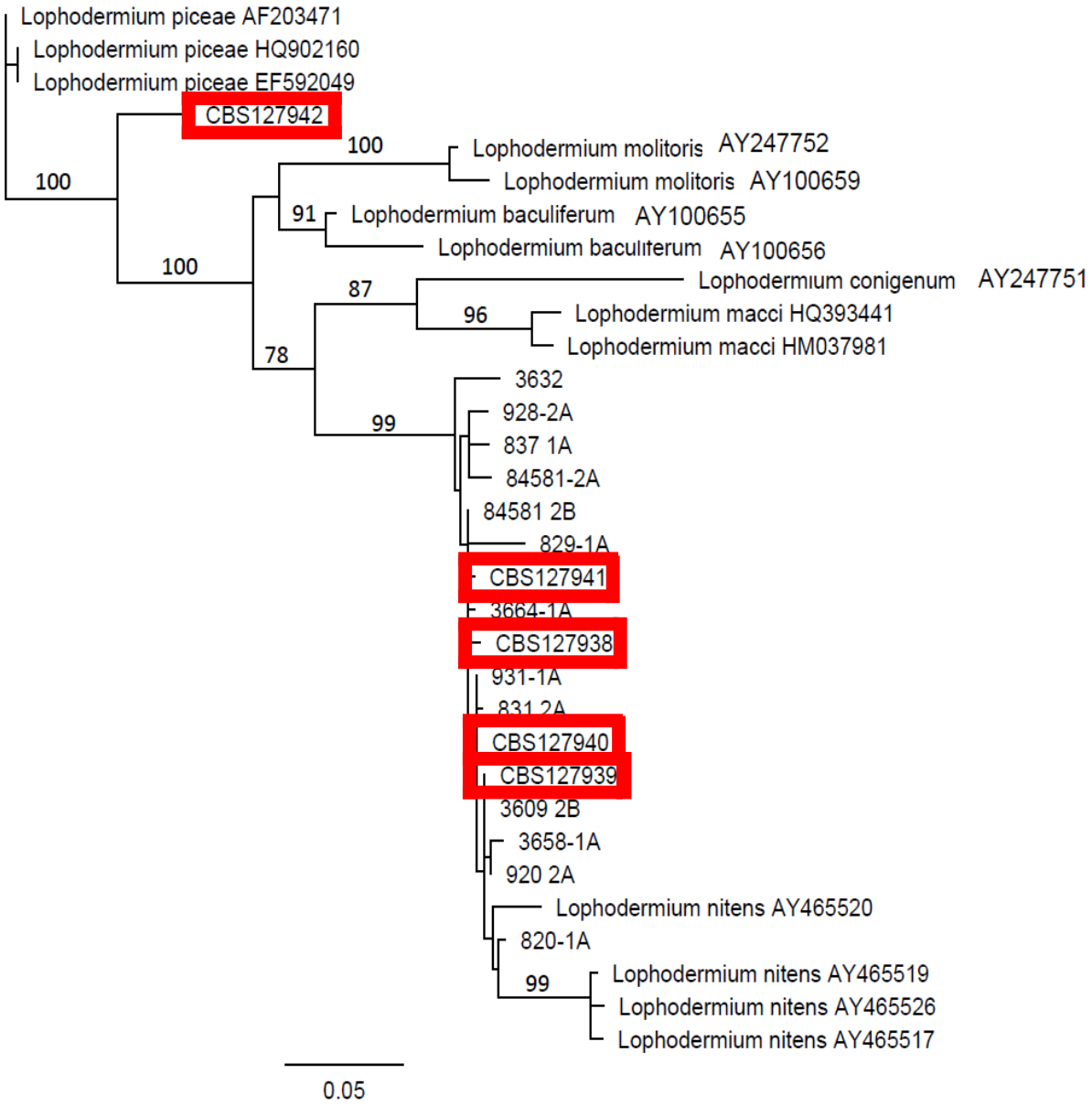

Figure 5. Phylogenetic relationships of selected pyrenophorol-producing strains.

Tree represents a PHYML maximum likelihood analysis of 32 taxa based on ITS rDNA sequence data (516 bp). Branch labels refer to PHYML bootstrap support values >70% based on 1000 replicates. Bar indicates nucleotide substitution per site. Target *Lophodermium* strains in red. Figure modified from Sumarah et al. 2014 (in review), courtesy A. Walker.

1.9 Fungal detection and identification

Fungi are difficult to identify, and current methods of identification include comparison of morphological features, ITS sequences (Schoch et al. 2012), immunoassays (Miller et al. 2009; Schmechel et al. 2008), chemical profiles (Frisvad et al. 2008) and lipid profiles (Zelles 1999).

Traditional methods of fungal detection and identification rely on microscopic and culture-based techniques. Microscopic identification of fungi generally requires sporulating structures, technical expertise and is time consuming. Culture based methods are biased towards the isolation of rapidly growing fungi and species present in environmental samples in high concentrations (Keswani et al. 2005). Some fungi may require specific conditions for growth and sporulation and may not grow on typically used media. Many fungi are unculturable as they are obligate biotroph, requiring a live plant host. Endophytes are particulary difficult to identify using traditional methods as many do not produce sporulating structures in culture. The identification of endophytes in conifer trees has traditionally been done through culturing surface sterilized needles (figure 6) and electron microscope techniques (Johnson et al. 1989). Non-fruiting isolates and the time required (8 weeks) to grow cultures are deterrents to traditional methods of identification. Contamination of competing faster growing phylloplane fungi also inhibits isolation (Miller et al. 2002) as endophytes are slow growing. Endophytes that produce secondary metabolites can be detected by the isolation of those compounds from needles (Miller et al. 2002).

Figure 6. Unidentified endophyte growing from surface sterilized pine needle.

1.10 Immunoassay based detection

Antibodies are glycoproteins secreted by specialized B lymphocytes found in plasma and extracellular fluids that serve as the first reponders for the adaptive immune system. Produced in response to molecules and organisms, they bind to an antigen with a high degree of affinity and specificity (Lipman et al. 2005). An antigen is a protein or carbohydrate that, when injected into animals, elicits the production of antibodies (Crowther 1995). Such antibodies react specifically with the antigen used and therefore can be used to detect that antigen. Antibodies are produced in response to antigenic stimuli, these are mostly protein in nature. Polyclonal antibodies are produced by several B cell lines of the humoral immunity of the adaptive immune system and recognize a variety of antigenic sites on the same macromolecule. Monoclonal antibodies are produced by single B cell lymphocyte types, making them very specific (Lipman et al. 2005). The decision to use poly or monoclonal antibodies depends on the intended use, monoclonals are

more specific but are much more expensive and can take over a year to be produced because of the purification steps involved to ensure their specificity. Polyclonal antibodies recognize several epitopes on target proteins making them less specific than monoclonal antibodies (Rosenberg 2006). Extensive analysis of the analytical bias of cross reactivity of ELISAs was done for environmental polyclonal immunoassays of *Alternaria alternata* (Schmechal et al. 2008). It was found by Schmechal et al. that the antibodies cross reacted extensively with other fungi, especially with species from the same family. A widespread sharing of antigens among fungi that necessitates the information on antibody specificity be tested and reported adequately to avoid any analytical bias (Schmechal et al. 2008) has been suggested. Closely related fungi have similar proteins in comparable concentrations, making cross reactivity more likely.

Immunoassays involve tests using antibodies as reagents. Enzyme-linked immunonsorbent assays (ELISAs) make use of enzymes attached to one of the reactants in an immunoassay, allowing the quantification of an antigen through the development of colour after the addition of a suitable substrate. ELISAs involve the stepwise addition and reaction of reagents to a solid phase bound substance, through incubation and separation of bound and free reagents using washing steps. An enzymatic reaction is utilized to produce colour and to quantify the reaction, through the use of an enzyme-labled reactant (Crowther 2005).

In an indirect ELISA, an antigen is attached to the solid phase microplate and is targeted by adding the developed primary 'detecting antibodies'. An enzyme-labeled conjugate secondary antibody is used to detect any bound primary antibodies. The addition of a chromophore such as

3,3′,5,5′-Tetramethylbenzidine substrate (TMB) causes a colour reaction through enzymatic catalysis, and this colour change is allowed to develop for a standardized amount of time (figure 7) and stopped with a pH change. The colour is quantified using a spectrophotometer at an appropriate wavelength and correlates to the amount of antigen bound to the microplate (Crowther 1995). The optical density (OD) or absorbance, is the intensity of light at a specific wavelength that has passed through a sample. Absorbance of a sample is porportional to the concentration of absorbing species in the sample.

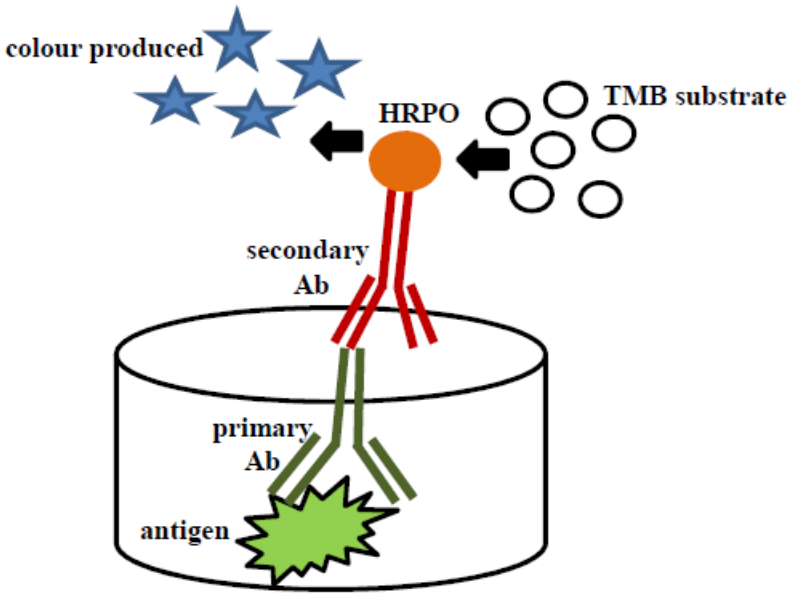

Figure 7. Indirect ELISA.

The antigen is directly bound to the solid phase microplate and is targeted by adding the primary antibody. An enzyme-labeled conjugate (HRPO) secondary antibody is used to detect any bound primary antibodies. The addition of a chromophore (TMB) causes a colour change when it reacts with the enzyme-labeled conjugate.

Enzyme-linked immunosorbent assays (ELISAs) have been used for the detection of fungi in house dust samples (Schmechel et al. 2008; Shi et al. 2011) and in environmental samples (Musgrave 1984; Sundaram et al. 1991). The endophyte *P. scopiformis* was successfully detected in pine needle samples using ELISA (Sumarah & Miller 2005; Miller et al. 2008; Sumarah et al. 2008; Miller et al. 2009). Miller et al. (2009) developed an ELISA that was specific and sensitive to detect the presence of *P. scopiformis* in needle and leaf samples. The method was developed using a polyclonal antibody and had a cell mass limit of detection at 60 ng/g of needle sample, sensitive enough to detect the endophyte in tree samples.

1.11 PCR based detections

The routine study of DNA became practical with the invention of polymerase chain reaction (PCR) by Kary Mullis in 1983. With PCR, it is possible to multiply a given DNA segment from complex genetic material millions of times in just a few hours using bench top equipment. A PCR reaction uses a thermocycler with defined temperature steps to heat and cool the reaction. Strands of DNA are separated using high temperatures, becoming templates for amplification. Heat stable DNA polymerase enzymatically assembles a new DNA strand from nucleotides. The single stranded DNA in the mixture is used as the template. Primers are starting points for DNA synthesis and are designed for selective amplification. PCR provided researchers with the ability to generate enough genetic material to study gene function and the effects of mutations, offering new possibilities for research, diagnostics and identification. Despite these advances, quantification of DNA remained a difficult undertaking until real-time kinetic monitoring of DNA was introduced. Higuchi et al. (1993) found that the relationship between the amount of target DNA and the amount of double stranded DNA PCR product generated after a specific

number of amplification cycles is linear. This observation formed the basis for real-time quantitative PCR (qPCR).

Real-time qPCR operates under the same principles as traditional PCR. The major difference is that during qPCR, the amount of PCR product is measured after each round of amplification instead of only at the end of the reaction (Logan et al. 2009). A fluorescent label is used to measure amplification products are they are produced. During amplification, a fluorescent dye binds to the accumulating DNA molecules and fluorescent values are recorded after each cycle of amplification. The fluorescent signal is directly proportional to the DNA concentration and the linear correlation between PCR product and fluorescence intensity is used to calculate the amount of template present at the beginning of each reaction (Logan et al. 2009). The point at which fluorescence is first detected as statistically significant above the baseline or background is called the cycle threshold or Ct value. This value is the most important parameter for qPCR. This threshold must be established to quantify the amount of DNA in samples. It is inversely correlated to the logarithm of the initial copy number (Logan et al. 2009). In absolute quantification assays, the numbers of a target gene are determined from a standard. Quantification of the unknown target template is calculated based on the comparison of Ct values of the target template to the constructed standard curve. Similarly, the crossing point (Cp) value is the point at which fluorescence of a sample rises above the background fluorescence in an absolute quantification analyses using Roche LightCycler 480 software. Crossing points of standards and unknown samples are the used to determine the concentration of target DNA.

There are two methods to perform qPCR: non-specific dye-based detection and probe-based specific detection. Dye-based detection is performed with the incorporation of a DNA binding dye in the PCR. Dyes are non-specific and bind to any double-stranded DNA generated during amplification, resulting in the emission of fluorescence. Probe-based quantitation uses sequence specific DNA-based fluorescent reporter probes. Sequence specific probes result in quantification of the DNA sequence of interest. The probes contain a fluorescent reporter dye and a quencher to prevent fluorescence (Houghton & Cockerill 2006; Logan et al. 2009).

There are many forms of qPCR probes. Hydrolysis probes, also known as TaqMan ® probes are especially effective. Developed by Roche Molecular Systems, the TaqMan method uses the 5'-3' exonuclease activity of *Thermus aquaticus* (*Taq*) DNA polymerase to cleave a labeled probe when it is hybridized to a complementary target. A flourophore is attached to the 5' end of the probe and a quencher to the 3' end. If no amplicon complementary to the probe is present, the probe remains intact and low fluorescence is detected. If the PCR results in a complementary target, the probe binds to it during each annealing step. The double strand specific 5'-3' nuclease activity of the *Taq* enzyme displaces the 5' end of the probe and then degrades it. This process releases the fluorophore and quencher into the solution, increasing fluorescence (figure 8) (Koch 2004; Filion 2012). TaqMan probes are a more expensive option that dye based probes and requires an additional conserved site within a short amplicon sequence to be present (Smith & Osbourn 2008) but this adds to the TaqMan's specificity. Multiple TaqMan probes and primer sets can be used in different qPCR assays to differentiate between closely related sequences. Probes labelled with different fluorescent tags can facilitate the development of multiplex qPCR assays, where different targets can be co-amplified and quantified in the same reaction (Smith &

Osbourn 2008). The identification of multiple regions on one short amplicon may not always be available for multiplex design.

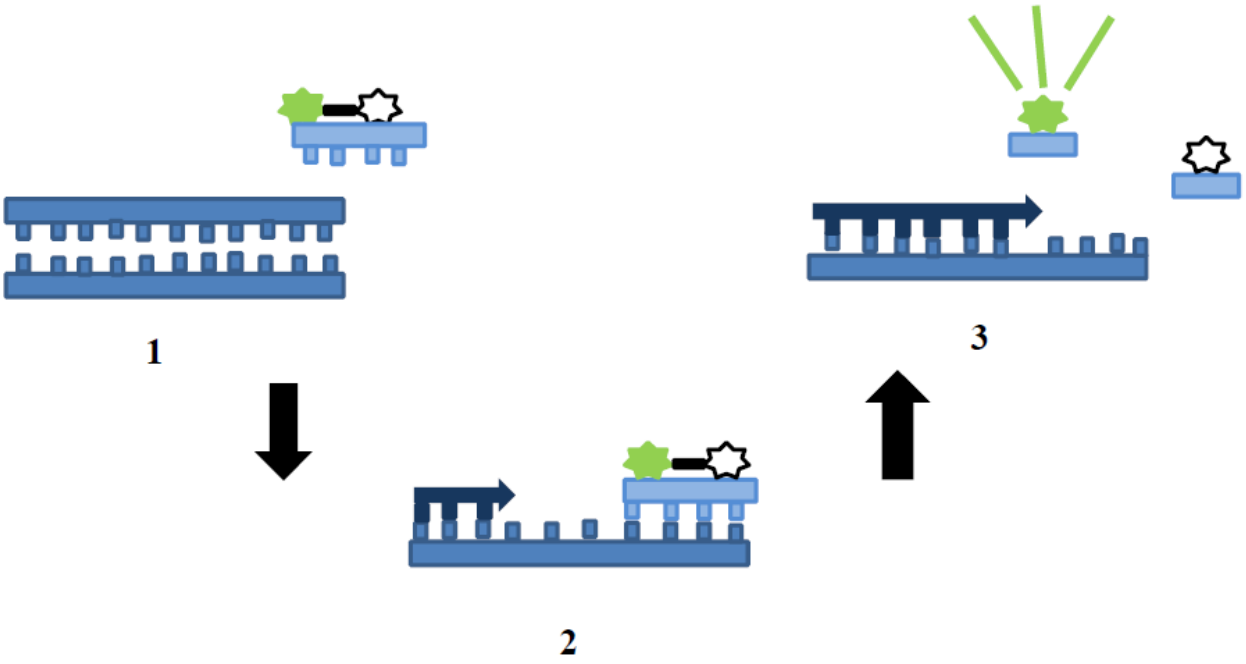

Figure 8. TaqMan ® hydrolysis probe.

1 The hydrolysis probe carries two fluorescent dyes in close proximity, the quencher dye supress the reporter fluorescent signal. 2 During the annealing phase, primers and probes specifically anneal to the target sequence. 3 As DNA polymerase extends the primer, it encounters the probe and cleaves it with its 5'-nuclease activity. The reporter dye is no longer quenched and emits fluorescent light.

DNA-based detection techniques are more suitable for fungi because these fungi may only exhibit few morphological characters. DNA-based techniques have been used to examine fungal

communities in a very diverse range of substrates such as roots, soil, air and animal tissues (Demeke et al. 2010; Tellenbach et al. 2010; Zhao et al. 2012; Muller et al. 2012).

Many studies detecting fungi using qPCR have been done on pure cultures in a laboratory setting and have not considered the effect of contaminants in environmental samples (Keswani et al. 2005). Reported inhibitors include humic and fulvic acids in soils, polyphosphates in fungi and plant polysaccharides (Keswani et al. 2005). There have been several studies focusing on the inhibitory compounds used in commercially available DNA isolation kits (Cruz-Perez et al. 2001; Cruz-Perez et al. 2001). Adding extraction steps to remove inhibitory compounds, adds complexity to sample preparation and is an additional source of variation in the data, especially in purification steps where much DNA can be lost. Sensitivity of a PCR assay depends on the physicochemical conditions of the reaction, the concentration of the DNA being analyzed and the PCR primers and probes (Bastien et al. 2008). These assays are dependent on DNA isolation, as even an optimized PCR assay will yield poor results if the DNA isolation was inadequate. PCR reactions require an extraction method that will efficiently lyse fungal cells and recover DNA suitable for amplification (Fredricks et al. 2005). Six commercial kits with different extraction methods (enzymatic lysis, nonenzymatic lysis, mechanical agitation) were compared for extraction yields of *Aspergillus* and *Candida* DNA (Fredricks et al. 2005). It was found that differences in yields were highly significant and optimal DNA isolation is dependent on the species. No single extraction method was found to be optimal for all organisms and can thus affect the results of qPCR assays (Fredericks et al. 2005.) The total amount of DNA in a template preparation includes DNA from the pathogen and its host. This affects the amount of free Mg^{2+} ions in the reaction and the enzymatic activity of the DNA polymerase (Bastien et al. 2008). A

high concentration of host DNA will affect the qPCR assay sensitivity (Bastien et al. 2008). When applying high reaction volumes, traces of inhibitory substances are often diluted to a subcritical level with the DNA.

1.12 Internal transcribed spacer region of fungal rDNA

For more than 20 years, the nuclear RNA cistron has been used for fungal diagnostics and phylogenetics (Schoch et al. 2011). The eukaryotic rRNA cistron consists of the 18S, 5.8S and 28S rRNA genes. These genes are transcribed as a unit by RNA polymerase I but posttranscriptional processes split the cistron, removing the two internal transcribed spacers (Schoch et al. 2011). These two spacers with the 5.8S unit are referred to as the ITS region (figure 9). Schoch et al. (2011) compared the ITS region with three genes that were being considered as the 'universal DNA barcode marker' for fungi. The ITS region was compared to the 28S nuclear ribosomal large subunit rRNA gene (LSU), the 18S nuclear ribosomal small subunit rRNA gene (SSU) and the largest subunit of rRNA polymerase II (RPB1) in a large scale multi-laboratory trial. Over 740 isolates were sampled from across the major fungal lineages (Schoch et al. 2011). The ITS region was chosen as the best barcoding gene because of its high PCR amplification and sequencing success rate (Schoch et al. 2011).

The ability to design qPCR primers and probes to target specific regions of DNA has led to a greater understanding of fungal ecology, plant-fungi interactions and fungal-fungal interactions (Atkins & Clark 2004). Quantitative PCR amplicons should be short (50-200 bps) with a GC content between 30-80%. A higher GC content will increase specificity of the reaction (Smith &

Osbourn 2008). Selecting target DNA using sequences allows primers and probes to be designed around conserved and variable regions (Atkins & Clark 2004). The ITS region has areas of conservation and variability, an ideal starting place for the development of specific primers and probes.

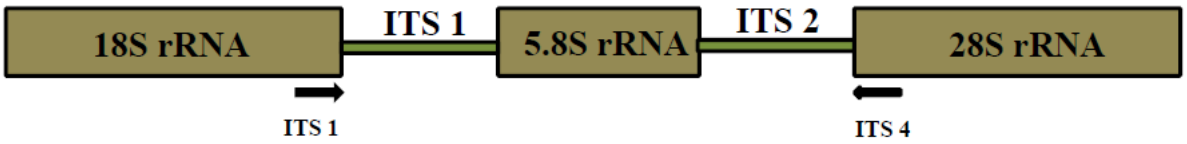

Figure 9. ITS ribosomal RNA gene region with primers.

Sequencing of the ITS region showed potential as a source of nuclear DNA characters for phylogenetic reconstruction in plants (Baldwin 1992). This region is used widely as an identifying marker in environmental sampling across many organisms from the fungal, plant and animal communities (Smith et al. 2007). Smith et al. (2007) examined the ITS variation in 68 closely related species of ectomycorrhizal fungi and detected a 40% variability rate, making intraspecific ITS variation more common than previously thought. In this study, primers and TaqMan probes were designed using the alignment of relevant ITS sequences from regionally associated fungi and the most closely related fungal species to the target species (figure 27, 28). Probes were centered on polymorphisms unique to the target ITS sequences. Assays have successfully been designed around single nucleotide polymorphisms (Mein et al. 2000; Prince et al. 2001).

OBJECTIVE

The purpose of this study was to develop enzyme-linked immunosorbent assays to detect the presence of three specific foliar endophytes in needle samples. Quantitative real-time PCR assays were also developed for three specific foliar endophytes. Comparisons were made between the two different assays as rapid detection methods for foliar endophytic fungi in conifer needles.

2. MATERIALS AND METHODS

2.1 Fungal endophyte strains

The fungi used in this study included *Lophodermium nitens* CBS 127938, CBS 127939, CBS 127941, *Lophodermium cf. piceae* CBS 127942 (Centraalbureau voor Schimmelcultures, Utrecht, Netherlands) which were isolated in Sussex NB (45˚ 54'46"N 64˚ 57'14"W; Sumarah et al. 2011). *Phialocephala scopiformis* DAOM 229536 (National Mycological Herbarium at the Department of Agriculture & Agri-Food Canada, Ottawa, ON) was isolated in Laurentian Park QC (47˚ 19'12"N 70˚ 49'48"W; Calhoun et al. 1992). Fungi used in cross reactivity testing included a strain of Xylariaceae CBS 120381, *Aspergillus fumigatus* CBS 545.65, *Penicillium chrysogenum* DAOM 234056, *Phialocephala compacta* CBS 507.94, *Phialocephala dimorphospora* CBS 300.12, *Phialocephala fortinii* FJ031031 and *Mycosphaerella* species CBS 121947. *Cladosporium cladosporioides* Paracel ID 1219013-1 was isolated by Paracel Laboratories Ltd., and identified by Don Belilse.

2.2 Enzyme-linked immunosorbent assay development

2.2.1 Protein isolation

Each strain of endophyte was grown in six 250-ml starter cultures consisting of 2% malt extract ME (Difco Laboratories, Lawrence KS) for one week incubated in the dark at 25˚C. The resulting cultures were macerated in ddH_2O under aseptic conditions and used to inoculate 2800 ml Fernbach flasks which were incubated for eight weeks in the dark at 25˚C before extra-cellular protein extraction.

The cultures for extracellular protein extraction were filtered with a metal strainer and the resulting mycelium rinsed with ddH_2O and frozen at -20 ℃. The filtrate was treated with 1 M NaCl, 10 ng/ml (0.007 mM) protease inhibitor cocktail (Sigma-Aldrich, Oakville ON) and 0.075 ng/ml (0.43 mM) phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, Oakville ON). The pH of the dissolved solution was adjusted to 9.0 with 2 M NaOH solution. Cellular debris and lipids were removed from the culture filtrate by centrifugation at 30,000 x g for 20 min at 4 ℃ and filtered through cheesecloth. Each culture filtrate was concentrated 40 X using a Masterflex L/S concentrator (Cole Parmer, Montreal QC) equipped with a Stedim Biotech (Sartorius, Gottingen DE) Vivavflow 200 with Hydrosart 10,000 kDa cut off membrane. Excess salt was removed from the supernatant using buffer exchanges performed by adding 900 ml of 20 mM Tris Base (pH 9) to 100 ml of concentrated supernatant. The buffer exchange was repeated two more times with the addition on 900 ml of 20 mM Tris Base to 100 ml of concentrated supernatant. Ten ng/ml of protease inhibitor cocktail was added and the concentrated filtrate was frozen at -20 ℃.

2.2.2 Protein concentration

Protein concentrations were determined using a Bradford Protein Assay (Bradford 1976). Protein samples were serially diluted with ddH_2O and 150 µl of each dilution were added to wells in a 96-well Nunc-immuno MaxiSorp plate (Sigma-Aldrich, Oakville ON). Blank wells consisted of 150 µl of ddH_2O. 150 µl of Quick Dye Bradford Reagent (Bio-Rad, Hercules CA) was added to the wells and incubated at room temperature for 10 min. Optical density was measured at 595 nm using a Spectramax 340PC microplate spectrophotometer (Molecular Device, Sunnyvale CA). A standard calibration curve based on dilutions of Bovine Serum

Albumin (BSA) (Sigma-Aldrich, Oakville ON) concentrations was used to determine the protein concentration of each sample.

2.2.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and gel staining

Protein samples were mixed with 5X SDS PAGE loading buffer in a 4:1 ratio, boiled for 5 min and then cooled on ice. Gels were placed into the electrophoresis tank and the samples were loaded onto a 10% acrylamide gel and. A SeeBlue® Plus2 Pre-Stained Standard marker (Invitrogen, Burlington ON) was loaded into the first lane for unknown protein molecular weight determination. Protein samples were separated by SDS-PAGE using the mini VE Vertical Electrophoresis System and Amersham Biosciences electrophoresis tank (GE Healthcare, Baie d'Urfe QC) powered by a Power Pac 1000 gel electrophoresis power supply (Bio-Rad, Mississauga ON). Resolution was achieved by using the Laemmli running buffer system (25 mM Tris,192mM glycine, 0.1 % SDS), with a voltage of 100 V for 20 min, followed by a voltage of 200 V for 80 min.

Coomassie staining was performed to visualize proteins (de St. Groth et al. 1963) having a total concentration greater than 50 ng. The gel was placed in Coomassie Brilliant Blue (CBB) stain reagent (Thermo Fisher Scientific, Waltham MA), incubated overnight at room temperature on a Thermolyne Aros 160 orbital shaker (Thermo Fisher Scientific, Waltham MA) set at 60 RPM. The next day, the CBB reagent was discarded and the gel was de-stained with 20 min changes of 20% ethanol solution and then rinsed with ultrapure water and scanned using a GS 800 densitometer (Bio-Rad, Mississauga ON).

A modified non-fixing silver staining procedure for polyacrylamide gels was performed to visualize proteins of low concentration (less than 50ng total protein). The gel was treated with a 50% ethanol: 5% acetic acid: 45% ddH_2O fixation solution and incubated for 30 min. The gel was then washed for 10 min in a 50% ethanol solution and rinsed with ddH_2O for 10 min, twice. The gel was sensitized with a freshly prepared 0.02% thiosulphate solution for 5 min and washed twice with ddH_2O. To stain the gel, it was immersed in a 0.1% silver nitrate solution for 60 min and was rinsed repeatedly with ddH_2O for 10 s each time. A developing solution of freshly prepared 0.04% formalin in 2% sodium carbonate was added to cover the gel for 10 s and then discarded. The developing solution was added again to cover the gel and was left until bands were visible (30 s-2 min). The developing solution was discarded and the gel was placed in a stopping solution of 5% acetic acid for 10 min. The gel was washed with ddH_2O and scanned with a GS 800 densitometer. Gels were stored in covered glass dishes in a 1% acetic acid solution.

2.2.4 Polyclonal antibody production

Polyclonal antibodies were produced in rabbits at Cedarlane Laboratories, Ltd. (Hornby, Ontario; meets the requirements of the Canadian Council on Animal Care). A pre-immune bleed was drawn and each protein extract was used to immunize two rabbits with 0.5 mg of concentrated protein (day 0), respectively. Rabbits were boosted 3 times at 21 day intervals (after 28, 47 and 66 days) with 0.5 mg of protein extract to increase the production of antibodies to the target protein. A test bleed was taken on day 66 and the terminal bleed was carried out on day 78.

L. nitens CBS 127938 was not chosen for polyclonal antibody development as progress had concurrently been made with other (qPCR) detection methods.

2.2.5 ELISA Development

2.2.5.1 Optimization

Indirect ELISA was developed by coating Nunc-Immuno Maxisorp 96-well plates with the respective target endophyte cells diluted in 50 mM carbonate-bicarbonate (pH 9.6) coating buffer (Sigma-Aldrich, St. Louis MO) ranging from 1.56-100 ng of cells per well. Blanks in this assay consisted of coating buffer. The antigen was incubated for four hours on a microplate shaker at room temperature. The plate was coated with 200 µl of sterile Blotto (10 g/l powdered non-fat dairy milk in TBS) and incubated overnight at 4 ℃. Plates were washed three times with TBST (pH 7.4) and 100 µl of primary antibody dilutions ranging from 1:1000 to 1:250,000 were added and left to incubate on a microplate shaker for one hour at room temperature. Plates were washed three times with TBST and 100 µl of 1:10,000 diluted secondary antibody anti-rabbit IgG peroxidase conjugate (Jackson Immuno Research, West Grove PA) was added to each well and incubated for one hour on a microplate shaker at room temperature. After being washed three times with TBST, 100 µl of TMB substrate (Sigma-Aldrich, St. Louis MO) was added to each well and allowed to develop for 30 min. An aliquot of 50 µl of 0.5 M H_2SO_4 was added to each well to stop development. The optical density of plates was immediately read at 450 nm with a Spectramax 340PC microplate spectrophotometer. ANOVA tests for limits quantification were calculated with SYSTAT 12 (Cranes Software, Chicago IL).

2.2.5.2 Cross reactivity testing

In addition to being tested against other target endophyte strains, each polyclonal antibody was tested for cross reactivity against *A. fumagatus* CBS 545.65 JN 943566*, C. cladosporioides* Paracel ID 1219013-1*, P. scopiformis* DAOM 229536 and a strain from Xylariaceae CBS 120381 as well as being tested against the other target endophyte strains that ELISA was developed for. Cells from 2% MEA plates were carefully removed, frozen at -20 ̊C for 48 hours and then freeze dried in a Labconco Freezone Freeze Dryer (Labconco, Kansas City MO). After freeze drying, cells were ground to a fine powder using a 5100 Mixer Mill (Spex SamplePrep, Metuchen NJ). Target endophyte cells were diluted from 10-500 ng in 50 mM carbonate-bicarbonate (pH 9.6) coating buffer and used to assess the cross reactivity of the primary antibodies diluted from 1:1,000-1:250,000. 60 ng of cells were incubated for four hours on a microplate shaker at room temperature. The plate was coated with 200 µl of sterile Blotto and incubated overnight at 4 ̊C. Plates were washed three times with TBST and 100 µl of primary antibody were added and left to incubate on a microplate shaker for one hour at room temperature. Plates were washed three times with TBST and 100 µl of 1:10,000 diluted secondary antibody anti-rabbit IgG peroxidase conjugate was added to each well and incubated for one hour on a microplate shaker at room temperature. After being washed three times with TBST, 100 µl of TMB substrate was added to each well and allowed to develop for 30 min. An aliquot of 50 µl of 0.5 M H_2SO_4 was added to each well to stop development. The optical density of plates was immediately read at 450 nm with a Spectramax 340PC microplate spectrophotometer.

2.2.5.3 Needle testing

The needle matrix effects of future tree samples to be tested were assessed by running assays with wells coated with 500 ng of freeze dried, ground pine needles and spiking the wells with 30-100 ng of target endophyte cells. Final OD values were compared with spiked wells on the same plate but without the addition of pine needles. Needles were added to each well and then spiked with the target endophyte and shaken on a microplate shaker for four hours at room temperature. The plate was coated with 200 µl of sterile Blotto and incubated overnight at 4 ̊C. Plates were washed three times with TBST and 100 µl of primary antibody dilutions of 1:56,000 were added and left to incubate on a microplate shaker for one hour at room temperature. Plates were washed three times with TBST and 100 µl of 1:10,000 diluted secondary antibody anti-rabbit IgG peroxidase conjugate was added to each well and incubated for one hour on a microplate shaker at room temperature. After being washed three times with TBST, 100 µl of TMB substrate was added to each well and allowed to develop for 30 min. An aliquot of 50 µl of 0.5 M H_2SO_4 was added to each well to stop development. The optical density of plates was immediately read at 450 nm with a Spectramax 340PC microplate spectrophotometer.

2.2.6 Test inoculations

Needle samples of white pine inoculated with target endophytes were tested using the optimized assays. Conifer seedlings were sprayed with an inoculum of a specific endophyte in a nursery setting and allowed to grow for 16 weeks. Approximately 100 mg of needles were taken from each seedling, freeze dried and ground into a powder.

To test each tree sample, needles were diluted in coating buffer and tested in duplicate wells at concentrations of 100 ng/well and 500 ng/well. The plate was coated with 200 µl of sterile Blotto and incubated overnight at 4 ℃. Plates were washed three times with TBST and 100 µl of primary antibody dilutions of 1:56,000 were added and left to incubate on a microplate shaker for one hour at room temperature. Plates were washed three times with TBST and 100 µl of 1:10,000 diluted secondary antibody anti-rabbit IgG peroxidase conjugate was added to each well and incubated for one hour on a microplate shaker at room temperature. After being washed three times with TBST, 100 µl of TMB substrate was added to each well and allowed to develop for 30 min. An aliquot of 50 µl of 0.5 M H_2SO_4 was added to each well to stop development. The optical density of plates was immediately read at 450 nm with a Spectramax 340PC microplate spectrophotometer.

In each test, the optimized (gave an OD of 1.00) amount of cells were used as positive controls to assess the performance of the assay. 40 ng/well for *L. cf. piceae* CBS 127942, 35 ng/well for *L. nitens* CBS 127941 and 60 ng/well for *L. nitens* CBS 127939. The assay results were analyzed if an acceptable positive control result was obtained. Samples with high absorbance values in both the 100 ng and 500 ng tests were rejected as an indication of dilution issues. Results were considered positive when the absorbance of the 500 ng sample was greater than the lowest absorbance value above 1 plus the mean absorbance value of half of the optimized target endophyte on that plate.

2.3 qPCR assay development

2.3.1 Fungal culture, growth conditions and DNA extraction

Fungal isolates used in the development of these assays were grown on 2% malt extract agar and incubated in the dark at 25 ̊C for 3 weeks prior to DNA extraction. For the purposes of primer and probe design, generation of standard curves and cross reactivity testing, DNA was extracted from 50 mg of mycelium from each *P. scopiformis* DAOM 229536, *L. nitens* CBS 127941 and *L. nitens* CBS 127939 culture with an UltraClean Microbial DNA Isolation Kit (MoBio, Carlsbad CA) according to the manufacturer's instructions. DNA concentration was measured using a NanoDrop ND1000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham MA). DNA was extracted from cultures for cross reactivity testing in the same described manner.

2.3.2 PCR amplification and sequencing

The internal transcribed spacer (ITS) region of ribosomal DNA is the formally acknowledged barcode marker for fungal species identification (Schoch et al. 2012). The complete ITS region of the extracted DNA was amplified by PCR using primers ITS5 GGAAGTAAAAGTCGTAACAAGG (White et al. 1990) or ITS-1F CTTGGTCATTTAGAGGAAGTAA (Gardes & Bruns 1993) and ITS4 TCCTCCGCTTATTGATATGC (White et al. 1990) synthesized by Integrated DNA Technologies (Integrated DNA Technologies, Coralville IA).

PCR amplification were performed in a 20 µl volume using 12.2 µl ddH_2O, 0.2 µl of Taq DNA polymerase (Invitrogen, Carlsbad CA), 2.0 µl of PCR buffer (Invitrogen, Carlsbad CA), 1.2 µl of 50 mM $MgCl_2$, 0.4 µl (C) dNTPs (Invitrogen, Carlsbad CA) , 1 µl of each primer and 2 µl of template DNA ranging in concentration from 7-10 ng/µl. PCR was conducted using a TECHNE TC-3000 thermocycler (Bibby Scientific, Staffordshire UK) using the following parameters: initial denaturation at 94 ℃ for 10 min followed by 40 cycles of denaturation at 94 ℃ for 15 s, annealing at 53 ℃ for 30 s, and extension at 72 ℃ for 90 s. Final extension was at 72 ℃ for 10 min. The full length amplification products were sequenced in both directions by McGill University and Génome Québec Innovation Centre (Montreal CA). Sequences were edited in Clustal X (Thompson et al 2002). The ITS sequences were trimmed to begin with the 'CATTA' motif and aligned using Geneious R6 (Biomatters Ltd., Auckland NZ). ITS sequences of the following fungal isolates were obtained from NCBI GenBank and included in the generated alignment: *Phialocephala fortinii* FJ031031, *P. scopiformis* DAOM 229536 CBS 507.94, *P. compacta* CBS 507.94, *P. dimorphospora* CBS 300.12 and *A. fumigatus* CBS 545.65.

2.3.3 Primer and TaqMan hydrolysis probe development

Quantitative PCR primers and TaqMan probes were designed in Primer3 (Untergasser et al 2012) and Geneious R6 (Biomatters Ltd., Auckland NZ) using the alignment of relevant ITS sequences, targeting 180 bp -200 bp regions in the ITS sequences containing sequence polymorphisms unique to *P. scopiformis* DAOM 229536 and *Lophodermium nitens* CBS 127941. Primers were designed around the probe. The parameters in table 1 were followed for the design of primers and probes to ensure optimal performance.

Table 1. Basic parameters of primer and probe design for the development of qPCR assays.

parameter	primer	probe
Base Pairs	15-30	18-30
G+C Content	30-80%	30-80%
Tm	58-60℃	8-10℃ higher than primers
*max amplicon size 200	*no runs of identical bps	*more Cs than Gs
*primer Tm equal	*no more than 2 Cs or Gs in last 5 bps at 3'	*polymorphic site in middle

Primers and probes (table 1) were synthesized by Integrated DNA Technologies (Coralville IA). The probes were labeled at the 5' end with the fluorescent reporter dye 6-carboxyflourescein (FAM) with the quencher dye 6-carboxy-tetramethylrhodamine (TAMRA) at the 3' end.

Quantitative PCR was performed in a Roche LightCycler 480 System (Roche Diagnostics, Basel SW) using a commercial master mix containing FastStart Taq DNA Polymerase, reaction buffer, dNTP mix and 6.4 mM $MgCl_2$ (LightCycler 480 Probes Master Mix, Roche Diagnostics, Basel SW). Each 20 µl reaction was prepared with 10 µl 2X master mix, 6 µl ddH_2O, 0.25 µl of each primer (20 µM), 1.5 µl probe (20 µM) and 2 µl DNA. Internal standard curve dilutions and non-template controls were run in each 96 well plate. Tenfold serial dilutions ranging from 8.6 fg-8.6 ng for *P. scopiformis* DAOM 229536, 6.1 ng-6.1 fg *L. nitens* CBS 127941 and 3.1 ng-3.1 fg *L. nitens* CBS 127939 of DNA extracted from culture samples were used as templates for generating internal standard curves, with each dilution measured in triplicate. Standard curves were generated with these ranges of values at least three times on three different days with

triplicate values in order to investigate reproducibility of the assays. qPCR conditions were as follows: an initial denaturation step at 95℃ for 10 min followed by 50 cycles of denaturation at 95℃ for 15 s, annealing at 60℃ for 15 s and extension at 40℃ for 30 s.

2.3.4 Specificity and extraction sensitivity testing

To test the specificity of these assays, other species of conifer endophytes and needle phylloplane fungi collected from New Brunswick were analyzed using the qPCR probes developed. DNA was extracted from approximately 50 mg of fungal mycelium obtained from pure cultures grown on 2% MEA with an UltraClean Microbial DNA Isolation Kit following the manufacturer's instructions. Each 20 µl reaction was prepared with 10 µl 2X master mix, 6 µl ddH_2O, 0.25 µl of each primer (20 µM), 1.5 µl probe (20 µM) and 2 µl DNA. qPCR parameters were as described earlier. DNA from each culture extract was run in a 96 well plate in triplicate with both positive and negative (non-template) controls. Each PCR primer and TaqMan probe were tested for specificity.

To explore any inhibitory effect that the conifer needle matrix would have on the extraction of fungal DNA or the qPCR reaction, sample spiking was conducted using cells from a culture of *P. scopiformis* DAOM 229536 grown on 2% MEA. *P. scopiformis* hyphae were flash frozen in liquid nitrogen for 60 s and then ground into a powder with sterilized mortar and pestle. *P. scopiformis* hyphae were carefully weighed and diluted in ddH_2O aliquots in tenfold dilutions ranging from 0.1 to 1 mg, in duplicate. To one of each duplicate, 100 mg of freeze dried needles from white pine were added. DNA extraction was then carried out under the same conditions and

qPCR conditions were as follows: an initial denaturation step at 95 ℃ for 10 min followed by 50 cycles of denaturation at 95 ℃ for 15 s, annealing at 60 ℃ for 15 s and extension at 40 ℃ for 30 s.

2.3.5 qPCR on inoculated tree samples

P. scopiformis DAOM 229536:

116 samples were taken from a 13 year old white spruce tree known to be infected with *P. scopiformis* DAOM 229536. Samples had been analysed for rugulosin and then were stored at -20 ℃ until tested for the presence of *P. scopiformis* DAOM 229536 DNA. DNA was extracted from 75 mg of each dried needle sample with an UltraClean Microbial DNA Isolation Kit according to the manufacturer's instructions. Needle samples were also taken from 291 spruce seedlings previously inoculated with *P. scopiformis* DAOM 229536 and DNA was extracted from 60 mg of dried sample as previously mentioned. qPCR reactions were set up in 10 µl reactions using 5 µl 2X master mix, 3 µl ddH_2O, 0.125 µl of each primer (20 µM), 0.75 µl probe (20 µM) and 1 µl DNA. Internal standard curve dilutions and non-template controls were run in each 96 well plate. Sample DNA concentrations were calculated based on internal standard curve values for each run. qPCR conditions were as follows: an initial denaturation step at 95 ℃ for 10 min followed by 50 cycles of denaturation at 95 ℃ for 15 s, annealing at 60 ℃ for 15 s and extension at 40 ℃ for 30 s.

L. nitens CBS 127941:

52 needle samples from white pine seedlings inoculated with this strain were tested for its presence using ELISA and then stored at -20℃ until tested by qPCR. DNA was extracted from 75 mg of each dried needle sample with an UltraClean Microbial DNA Isolation Kit according to the manufacturer's instructions. qPCR reactions were set up in 10 µl reactions using 5 µl 2x master mix, 3 µl ddH_2O, 0.125 µl of each primer (20 µM), 0.75 µl probe (20 µM) and 1 µl DNA. Internal standard curve dilutions and non-template controls were run in each 96 well plate. Sample DNA concentrations were calculated based on internal standard curve values. qPCR conditions were as follows: an initial denaturation step at 95℃ for 10 min followed by 50 cycles of denaturation at 95℃ for 15 s, annealing at 60℃ for 15 s and extension at 40℃ for 30 s.

L. nitens CBS 127939:

100 needle samples from white pine seedlings inoculated with this strain were tested for its presence using ELISA and then stored at -20℃ until tested by qPCR. DNA was extracted from 75 mg of each dried needle sample with an UltraClean Microbial DNA Isolation Kit according to the manufacturer's instructions. qPCR reactions were set up in 10 µl reactions using 5 µl 2x master mix, 3 µl ddH_2O, 0.125 µl of each primer (20 µM), 0.75 µl probe (20 µM) and 1 µl DNA. Internal standard curve dilutions and non-template controls were run in each 96 well plate. Sample DNA concentrations were calculated based on internal standard curve values. qPCR conditions were as follows: an initial denaturation step at 95℃ for 10 min followed by 50 cycles of denaturation at 95℃ for 15 s, annealing at 60℃ for 15 s and extension at 40℃ for 30 s.

RESULTS

3.1 Protein concentration

The Bradford assay with a BSA standard was used to determine the amount of extracellular protein extracted from cultures. From the *L. nitens* CBS 127938 culture, 13.65 mg of protein was isolated. From the *L. nitens* CBS 127939 culture, 12.44 mg of protein was isolated. Protein concentrations for *L. nitens* CBS 127941 and *L. cf. piceae* CBS 127942 were approximately 10 mg each.

3.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) staining

Extracted proteins were run on an SDS-PAGE gel and proteins were visualized with a modified non-fixing silver staining procedure for polyacrylamide gels for proteins (figures 10-13). Proteins were visualized to ensure the presence of possible major antigens to be used in antibody development. *L. cf. piceae* CBS 127942 extracellular protein extract showed major bands at 35 kDa (figure 10). *L. nitens* CBS 127941 extracellular protein extract showed major bands at 45 kDa (figure 11). *L. nitens* CBS 127939 extracellular protein extract showed bands at 90 kDa (figure 12). *L. nitens* CBS 127938 extracellular protein extract showed bands at 90 kDa and 45 kDa (figure 13). Minor bands occurred with all four protein extracts at around 20 kDa and 36 kDa which may explain some of the cross reactivity observed between the closely related strains (figures 10-13, & 22-25). In *L. cf. piceae* CBS 127942, *L. nitens* CBS 127938 and *L. nitens* CBS 127939 bands were seen at 90 kDa (figure 10, 12, 13).

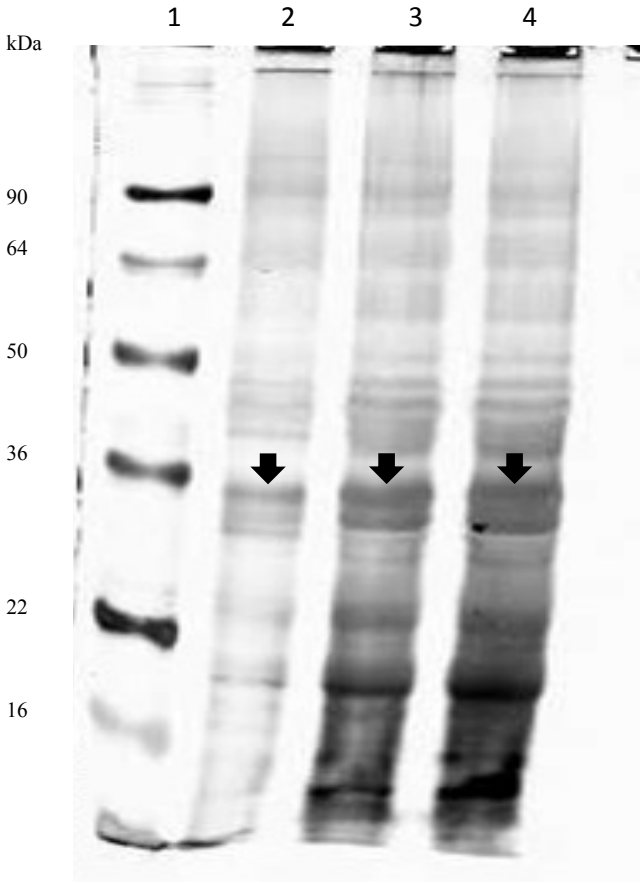

Figure 10. SDS-PAGE non-fixing silver stain of extracellular proteins from *Lophodermium cf. piceae* CBS 127942.

Lane 1: molecular weight marker (5 µl)
Lane 2*: L. cf. piceae* CBS 127942 extracellular protein extract (1 µl)
Lane 3*: L. cf. piceae* CBS 127942 extracellular protein extract (2 µl)
Lane 4: *L. cf. piceae* CBS 127942 extracellular protein extract (5 µl)
Arrows indicate bands of possible antigenic protein

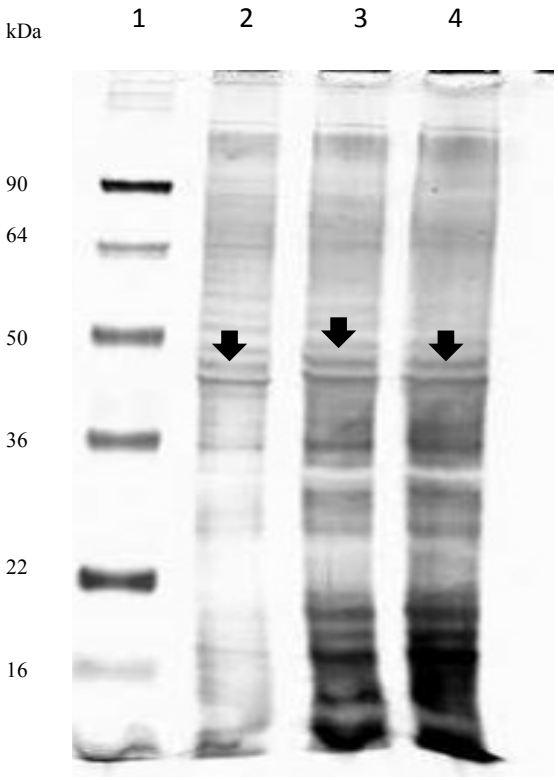

Figure 11. SDS-PAGE non-fixing silver stain of extracellular proteins from *Lophodermium nitens* CBS 127941.

Lane 1: molecular weight marker (5 µl)
Lane 2*: L. nitens* CBS 127941 extracellular protein extract (1 µl)
Lane 3*: L. nitens* CBS 127941 extracellular protein extract (2 µl)
Lane 4: *L. nitens* CBS 127941 extracellular protein extract (5 µl)
Arrows indicate bands of possible antigenic protein

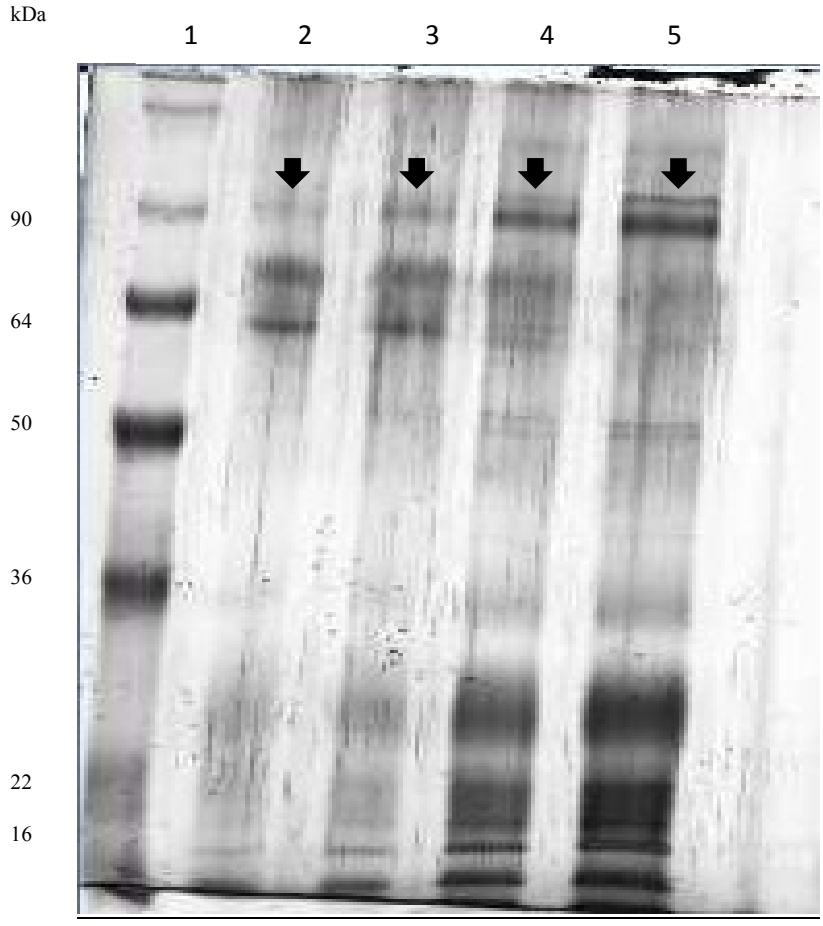

Figure 12. SDS-PAGE non-fixing silver stain of extracellular proteins from *Lophodermium nitens* CBS 127939.

Lane 1: molecular weight marker (5 µl)
Lane 2*: L. nitens* CBS 127939 extracellular protein extract (1 µl)
Lane 3*: L. nitens* CBS 127939 extracellular protein extract (2 µl)
Lane 4: *L. nitens* CBS 127939 extracellular protein extract (5 µl)
Lane 5*: L. nitens* CBS 127939 extracellular protein extract (7 µl)
Arrows indicate bands of possible antigenic protein

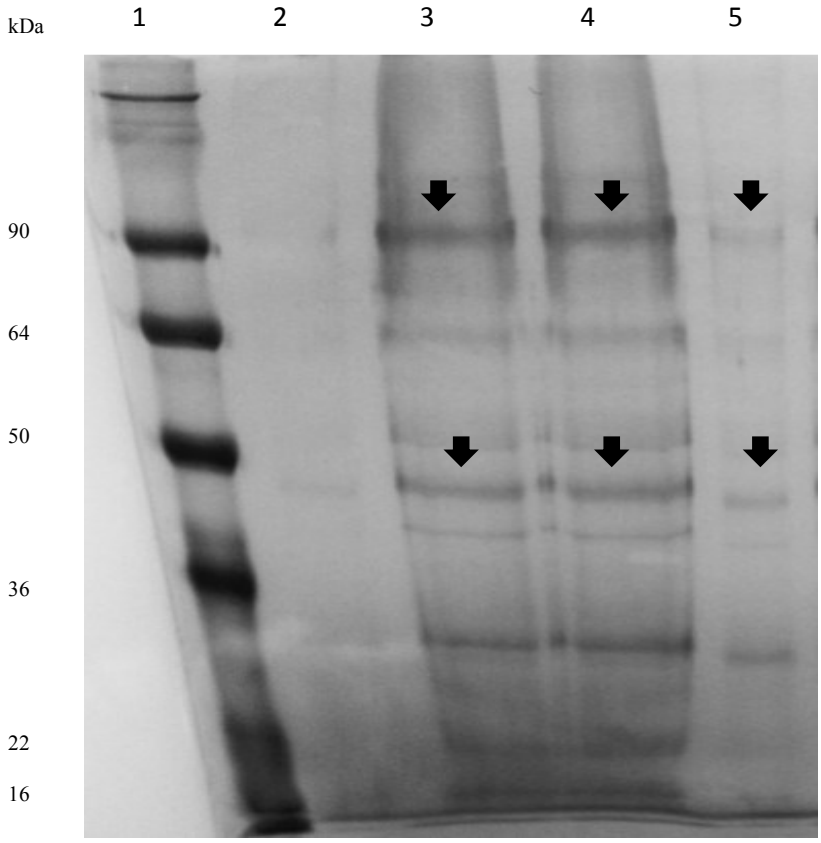

Figure 13. SDS-PAGE non-fixing silver stain of extracellular proteins from endophyte *Lophodermium nitens* CBS 127938.

Lane 1: molecular weight marker (5 µl)
Lane 2*: L. nitens* CBS 127938 extracellular protein extract (1 µl)
Lane 3*: L. nitens* CBS 127938 extracellular protein extract (5 µl)
Lane 4: *L. nitens* CBS 127938 extracellular protein extract (5 µl)
Lane 5*: L. nitens* CBS 127938 extracellular protein extract (2 µl)
Arrows indicate bands of possible antigenic protein

3.3 Polyclonal antibody production

Rabbit polyclonal antibodies (RpAbs) were produced by inoculating and boosting rabbits with their respective target antigens *L. nitens* CBS 127941*, L. nitens* CBS 127939 and *L. cf. picaea* CBS 127942. The protein extract for *L. nitens* CBS 127938 did not proceed to RpAb production.

3.4 ELISA development

3.4.1 Optimization

Experimental conditions were explored to optimize the RpAbs from rabbit sera using indirect ELISA methods. Optimal assay conditions (OD value~1) were determined for each RpAb by coating the microplate wells with endophyte cells at concentrations of 40 ng/well for *L. cf. piceae* CBS 127942 (figure 14), 35 ng/well for *L. nitens* CBS 127941(figure 15) and 60 ng/well for *L. nitens* CBS 127939, respectively (figure 16). A 1:56,000 dilution of primary RpAb and development with 1:10,000 diluted secondary antibody anti-rabbit IgG peroxidase conjugate was found to be optimal for each assay. Each optimized assay demonstrated a linear response over a range of target cell concentrations.

ELISA development conditions were optimal when the plate was coated with 200 µl of sterile Blotto and incubated overnight at 4 ℃. Plates were washed three times with TBST and 100 µl of primary antibody dilutions of 1:56,000 were added and left to incubate on a microplate shaker for one hour at room temperature. Plates were washed three times with TBST and 100 µl of 1:10,000 diluted secondary antibody anti-rabbit IgG peroxidase conjugate was added to each well and incubated for one hour on a microplate shaker at room temperature. After being washed

three times with TBST, 100 µl of TMB substrate was added to each well and allowed to develop for 30 min. An aliquot of 50 µl of 0.5 M H_2SO_4 was added to each well to stop development and the optical density of plates was immediately read at 450 nm with a Spectramax 340PC microplate spectrophotometer.

Limit of detection (LOD) was estimated for each assay .The experiments done did not determine the limit of detection i.e. the lowest value tested remained statistically significant from the blanks (ANOVA, followed by Tukey HSD). For this reason the LOQ was estimated as the second lowest value, 50 ng mycelium for each case. Absorbance values were highly correlated indicating that the linearity of the assay was not affected by presence of the needle matrix.

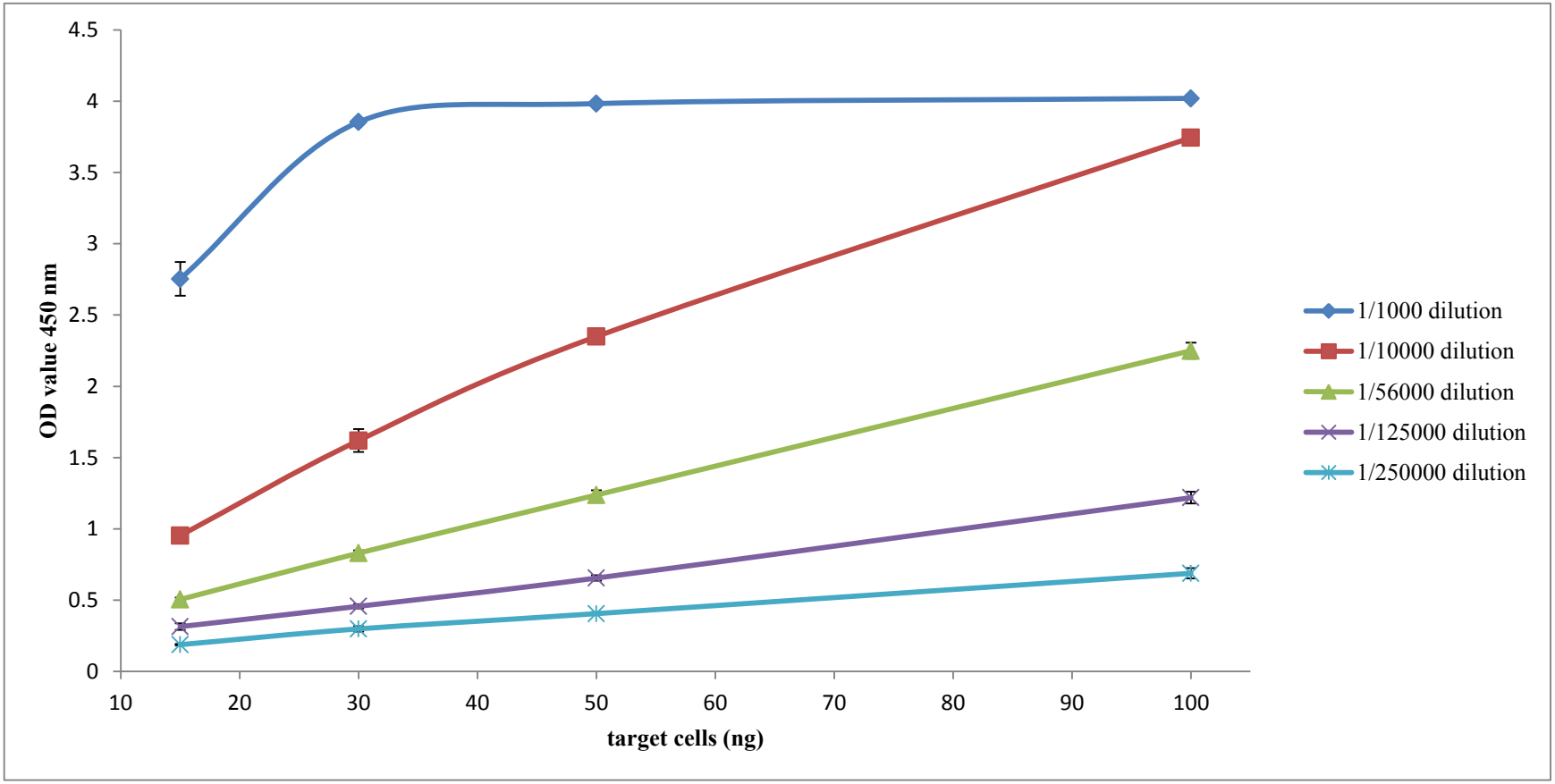

Figure 14. Indirect ELISA for *Lophodermium cf. piceae* CBS 127942.

Plate coated with range of *L. cf. piceae* CBS 127942 cells from pure culture; blocking: 4hrs with Blotto; developing antibody: multiple dilutions; detection: 1:10,000X HRPO. Data points represent an average from a portion of the same sample of mycelium done in 9 replicate microplate wells. Where not visible, standard error bars are within the data point symbols.

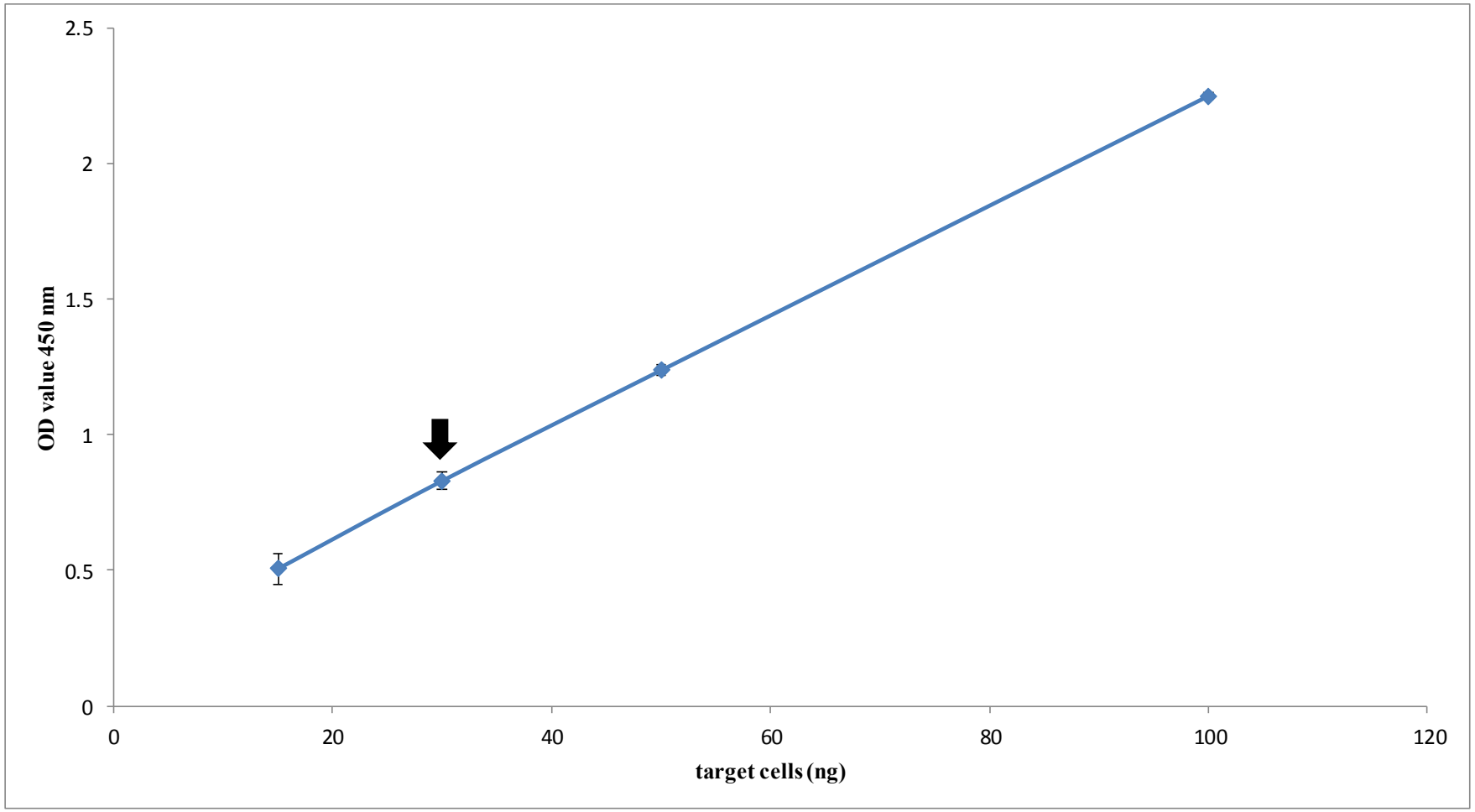

Figure 15. Final indirect ELISA for *Lophodermium cf. piceae* CBS 127942.

Plate coated with range of *L. cf. piceae* CBS 127942 cells from pure culture; blocking: 4hrs with Blotto; developing antibody: 1:56,000X polyclonal antibody; detection: 1:10,000X HRPO. Data points represent an average from a portion of the same sample of mycelium done in 9 replicate microplate wells. Where not visible, standard error bars are within the data point symbols. $R^2 = 0.9999$. Arrow indicates estimated LOD (30 ng cells).

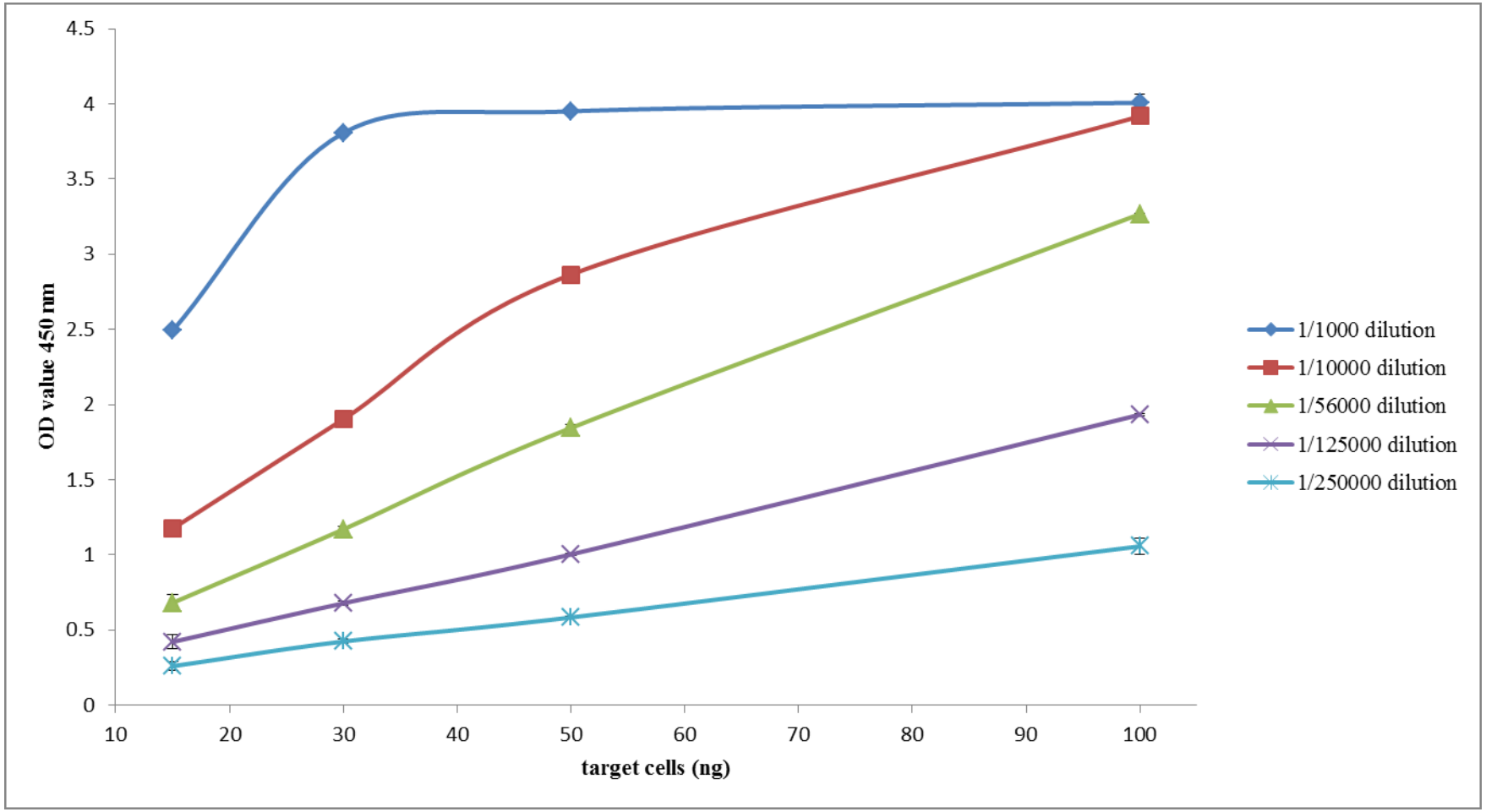

Figure 16. Indirect ELISA for *Lophodermium nitens* CBS 127941.

Plate coated with range of *L. nitens* CBS 127941 cells from pure culture; blocking: 4hrs with Blotto; developing antibody: multiple dilutions; detection: 1:10,000X HRPO. Data points represent an average from a portion of the same sample of mycelium done in 9 replicate microplate wells. Where not visible, standard error bars are within the data point symbols.

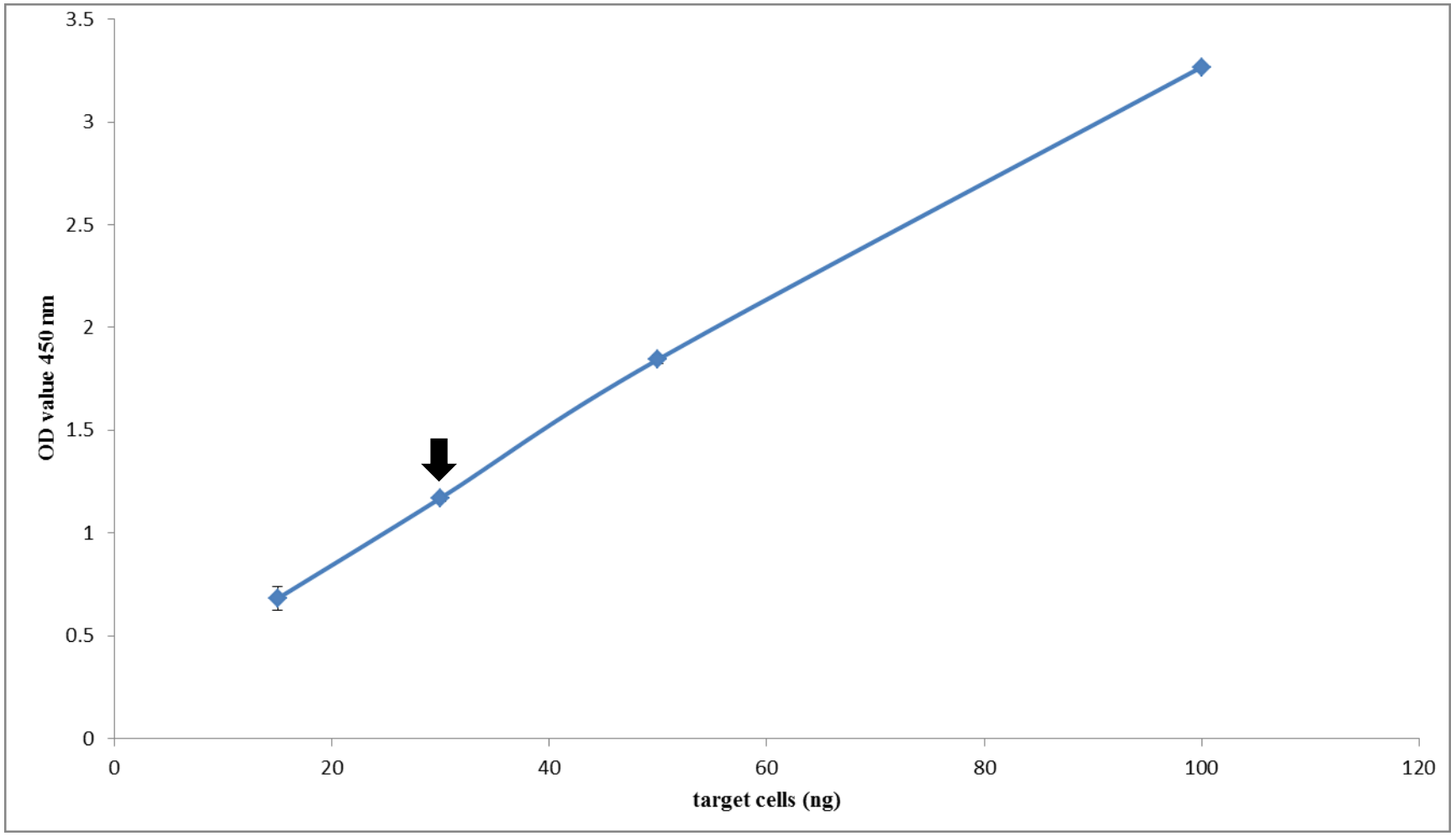

Figure 17. Final indirect ELISA for *Lophodermium nitens* CBS 127941.

Plate coated with range of *L. nitens* CBS 127941 cells from pure culture; blocking: 4hrs with Blotto; developing antibody: 1:56,000X polyclonal antibody; detection: 1:10,000X HRPO. Data points represent an average from a portion of the same sample of mycelium done in 9 replicate microplate wells. Where not visible, standard error bars are within the data point symbols. $R^2 = 0.9983$. Arrow indicates estimated LOD (30 ng cells).

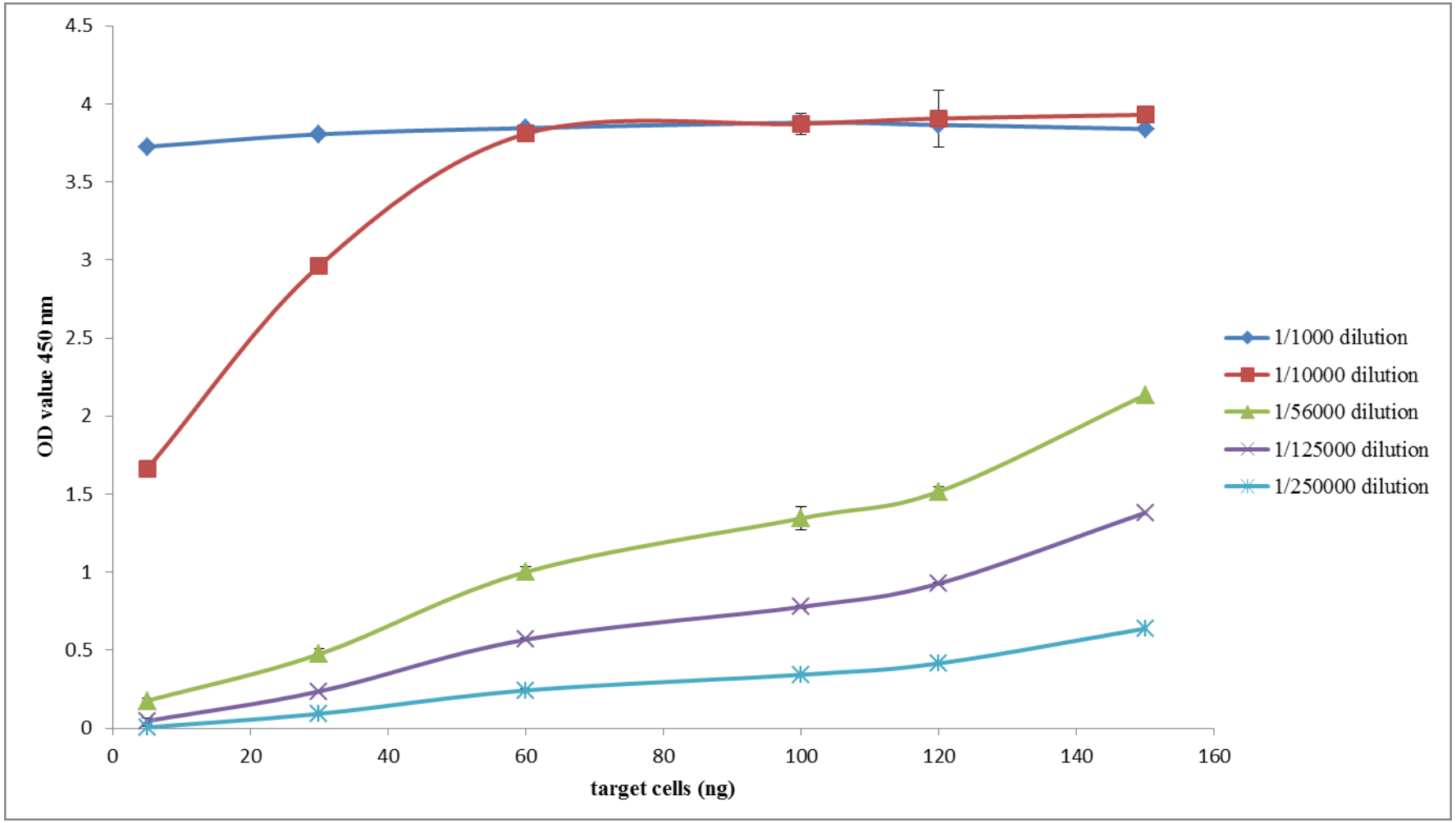

Figure 18. Indirect ELISA for *Lophodermium nitens* CBS 127939.

Plate coated with range of *L. nitens* CBS 127939 cells from pure culture; blocking: 4hrs with Blotto; developing antibody: multiple dilutions; detection: 1:10,000X HRPO. Data points represent an average from a portion of the same sample of mycelium done in 9 replicate microplate wells. Where not visible, standard error bars are within the data point symbols.

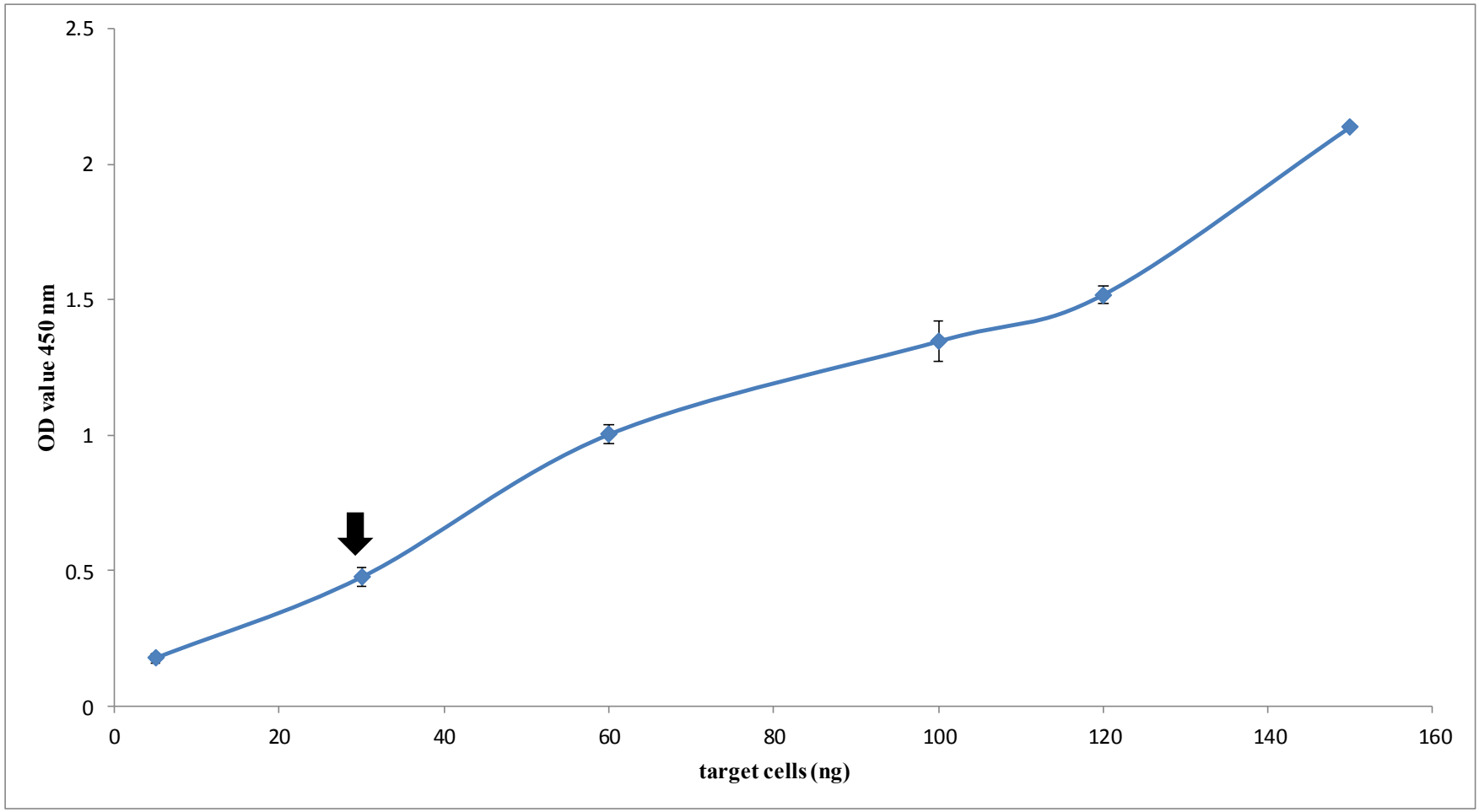

Figure 19. Final indirect ELISA for *Lophodermium nitens* CBS 127939.

Plate coated with range of *L. nitens* CBS 127939 cells from pure culture; blocking: 4hrs with Blotto; developing antibody: 1:56,000X polyclonal antibody; detection: 1:10,000X HRPO. Data points represent an average from a portion of the same sample of mycelium done in 9 replicate microplate wells. Where not visible, standard error bars are within the data point symbols. $R^2 = 0.9823$. Arrow indicates estimated LOD (30 ng cells).

3.4.2 Cross reactivity testing

Each polyclonal antibody was tested for cross reactivity against *A. fumigatus* CBS 545.65*, C. cladosporioides* Paracel ID 1219013-1*, P. scopiformis* DAOM 229536*, L. nitens* CBS 127938 and a Xylariaceae sp. CBS 120381. Relative cross reactivity to the phylloplane fungi (*C. cladosporioides* & *P. chrysogenum*) and *P. scopiformis* was minimal across antibody and cell dilutions for each assay (figures 20-25).

There was greater cross reactivity between the *Lophodermium* strains, as would be expected because they are closely related. The *L. cf. piceae* CBS 127942 Ab gave a clear response to the target endophyte (figure 20, 21) as did *L. nitens* CBS 127939 (figure 24, 25). The avidity of the *L. nitens* CBS 127941 Ab was greater for *L. cf. piceae* CBS 127942 (figure 22, 23).

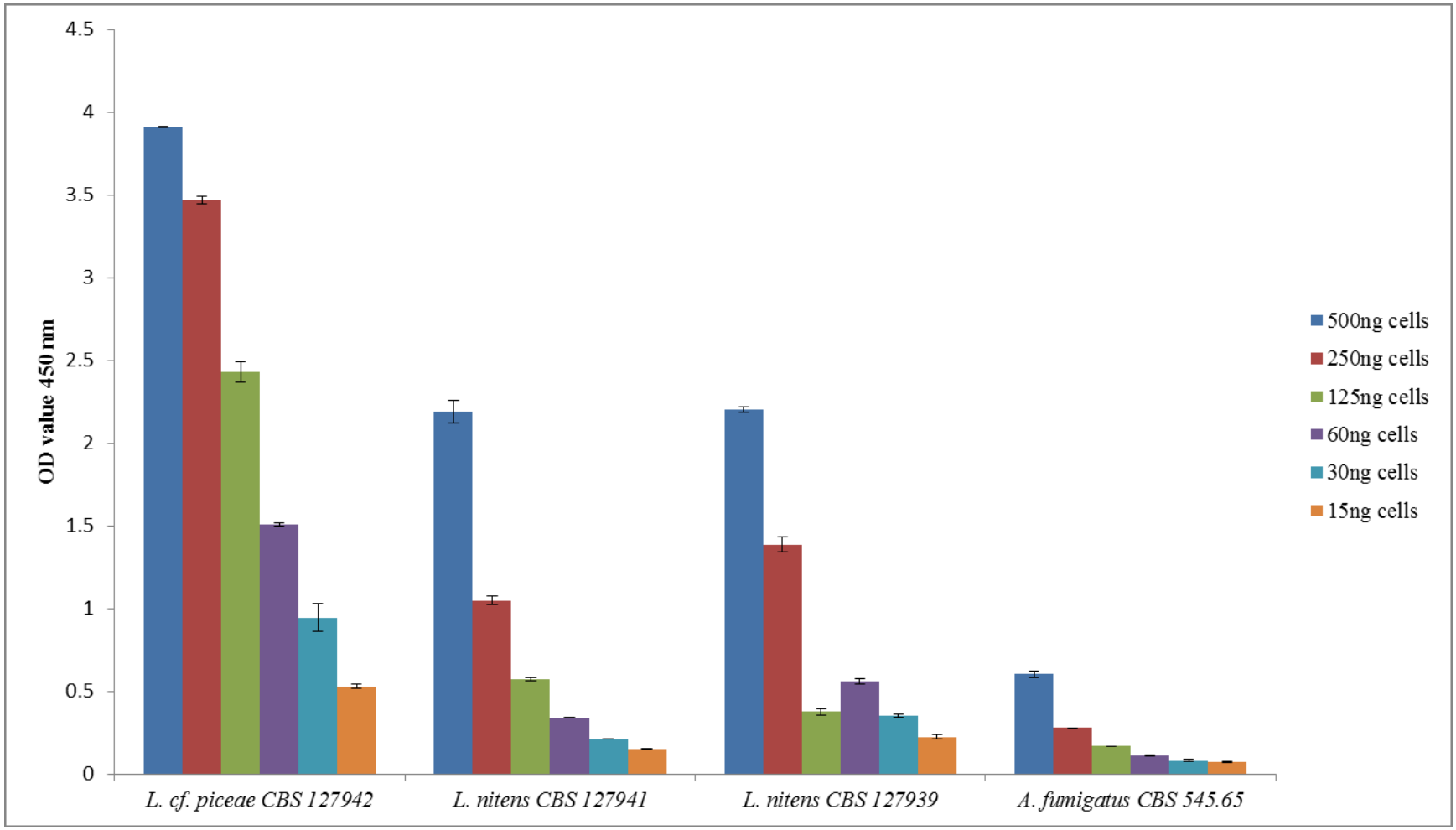

Figure 20. Cross reactivity of *Lophodermium cf. piceae* CBS 127942 assay.

Plate coated with range of fungal cells from pure culture; blocking: 4hrs with Blotto; developing antibody: 1:56,000X *Lophodermium cf. piceae* CBS 127942 polyclonal antibody; detection: 1:10,000X HRPO. Data points represent an average from a portion of the same sample of mycelium done in 9 replicate microplate wells. Error bars represent standard error.

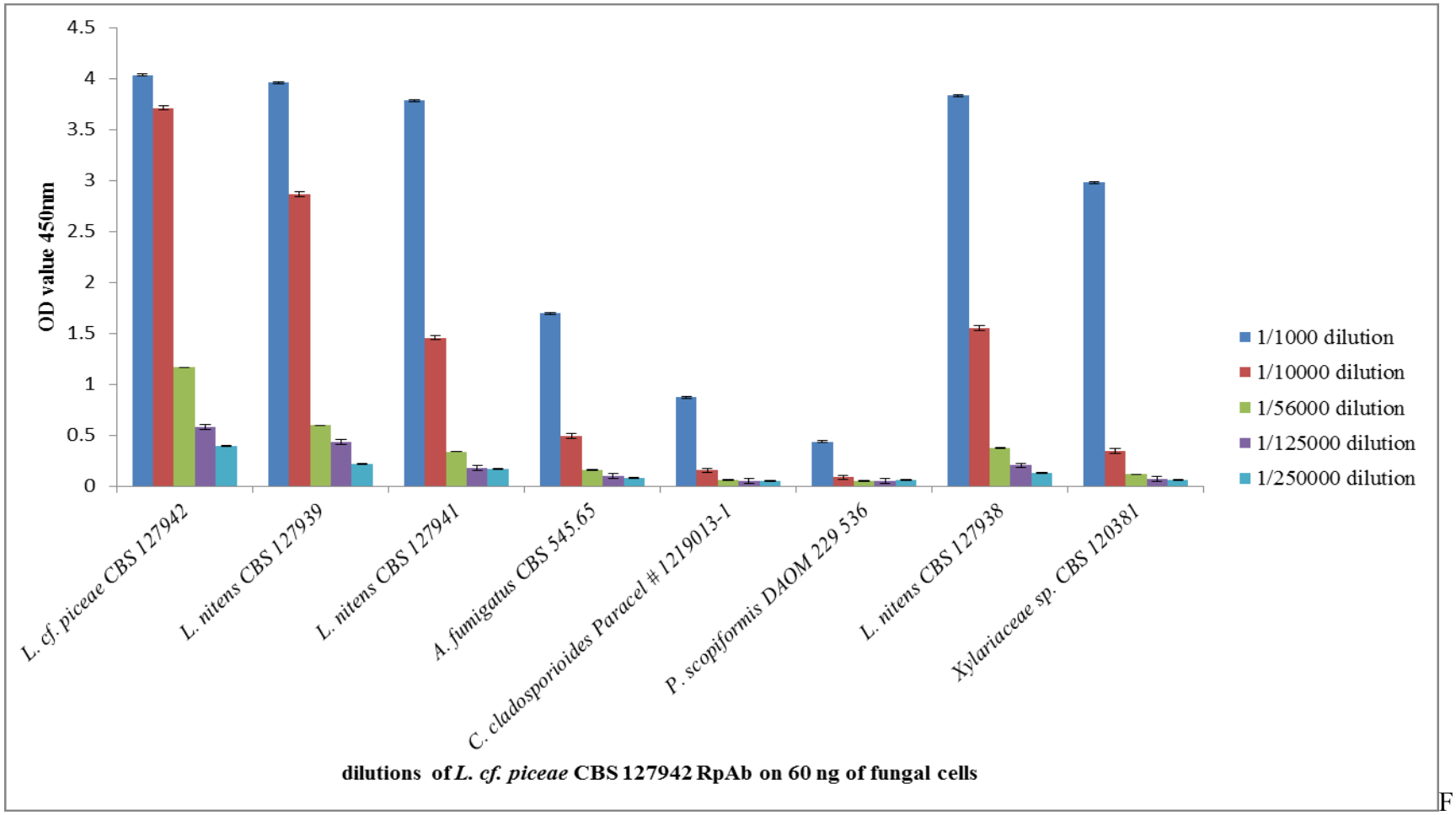

Figure 21. Cross reactivity of *Lophodermium cf. piceae* CBS 127942 assay.

Plate coated with 60 ng of fungal cells from pure culture; blocking: 4hrs with Blotto; developing antibody: dilutions of *Lophodermium cf. piceae* CBS 127942 polyclonal antibody; detection: 1:10,000X HRPO. Data points represent an average from a portion of the same sample of mycelium done in 9 replicate microplate wells. Error bars represent standard error.

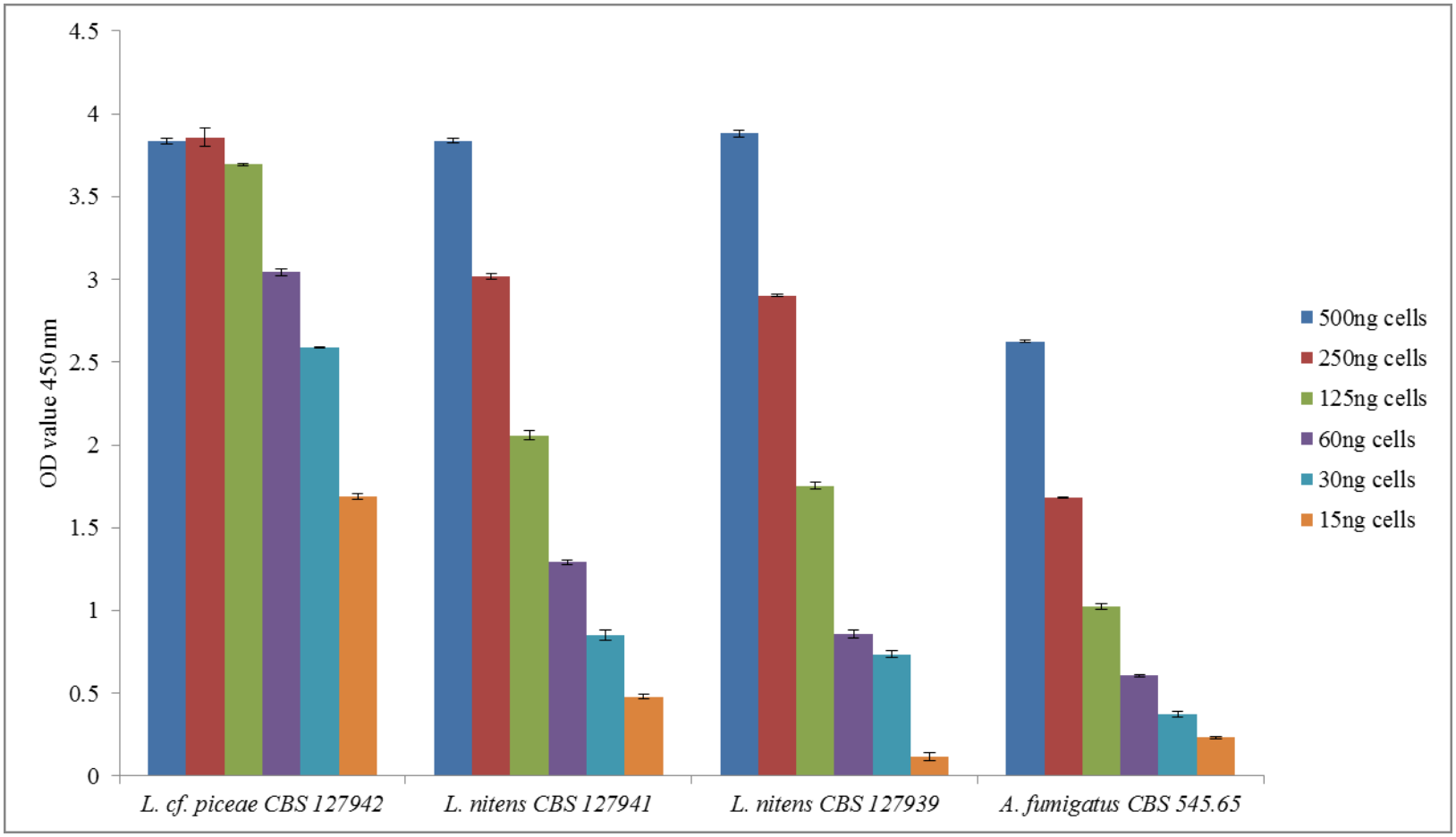

Figure 22. Cross reactivity of *Lophodermium nitens* CBS 127941 assay.

Plate coated with range of fungal cells from pure culture; blocking: 4hrs with Blotto; developing antibody: 1:56,000X *Lophodermium nitens* CBS 127941 polyclonal antibody; detection: 1:10,000X HRPO. Data points represent an average from a portion of the same sample of mycelium done in 9 replicate microplate wells. Error bars represent standard error.

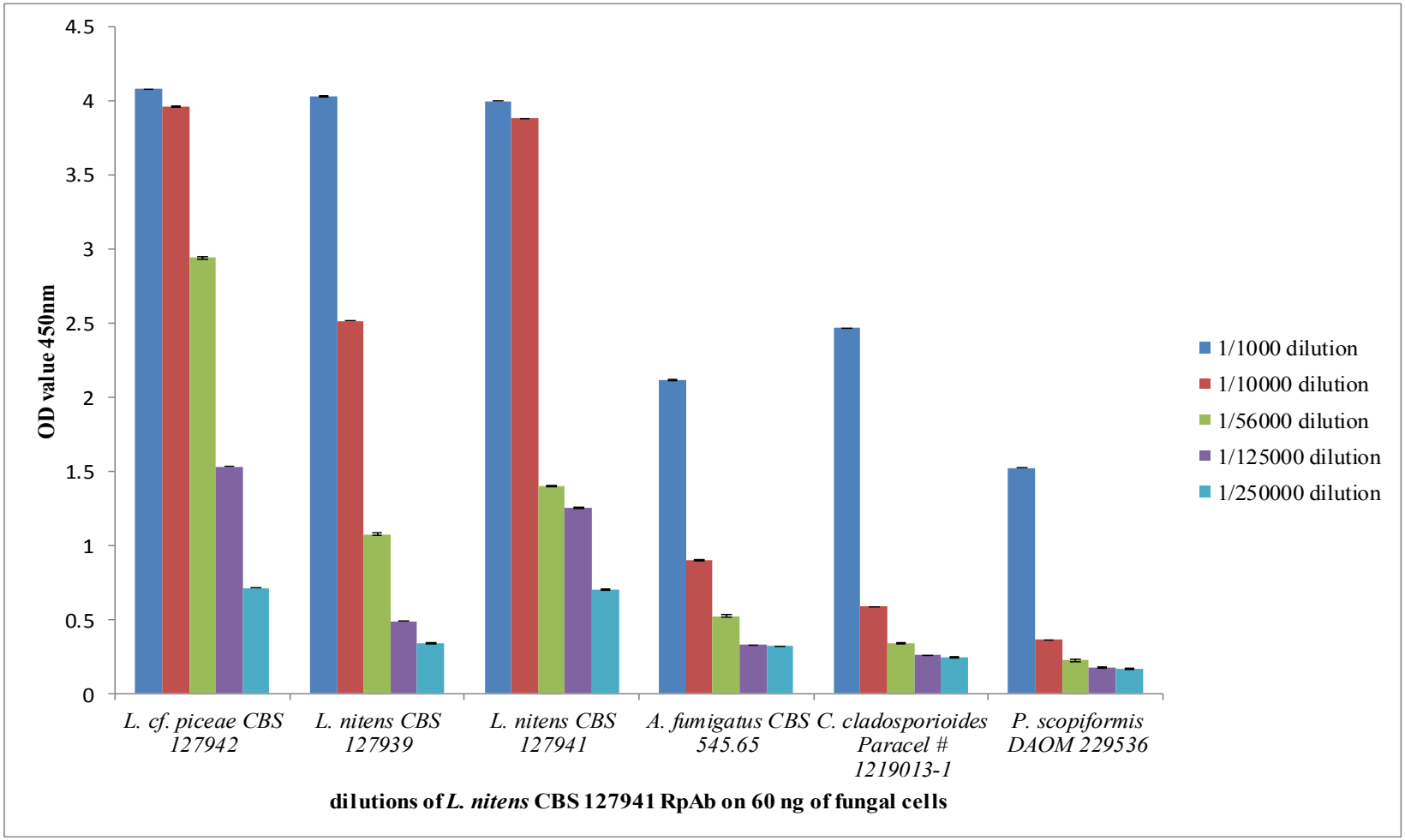

Figure 23. Cross reactivity of *Lophodermium nitens* CBS 127941 assay.

Plate coated with 60 ng of fungal cells from pure culture; blocking: 4hrs with Blotto; developing antibody: dilutions of *Lophodermium cf. piceae* CBS 127941 polyclonal antibody; detection: 1:10,000X HRPO. Data points represent an average from a portion of the same sample of mycelium done in 9 replicate microplate wells. Error bars represent standard error.

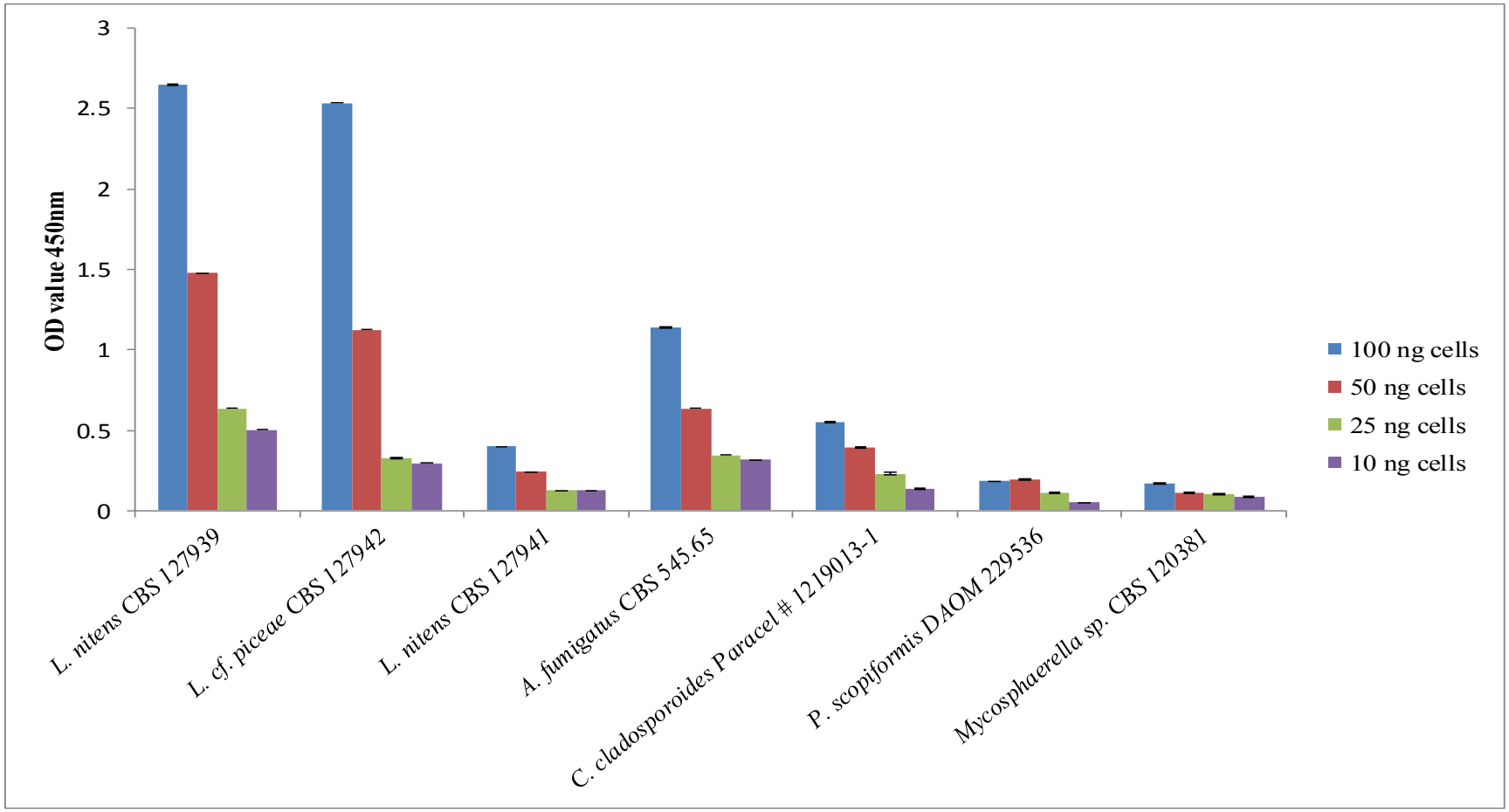

Figure 24. Cross reactivity of *Lophodermium nitens* CBS 127939 assay.

Plate coated with range of fungal cells from pure culture; blocking: 4hrs with Blotto; developing antibody: 1:56,000X *Lophodermium nitens* CBS 127939 polyclonal antibody; detection: 1:10,000X HRPO. Data points represent an average from a portion of the same sample of mycelium done in 9 replicate microplate wells. Error bars represent standard error.

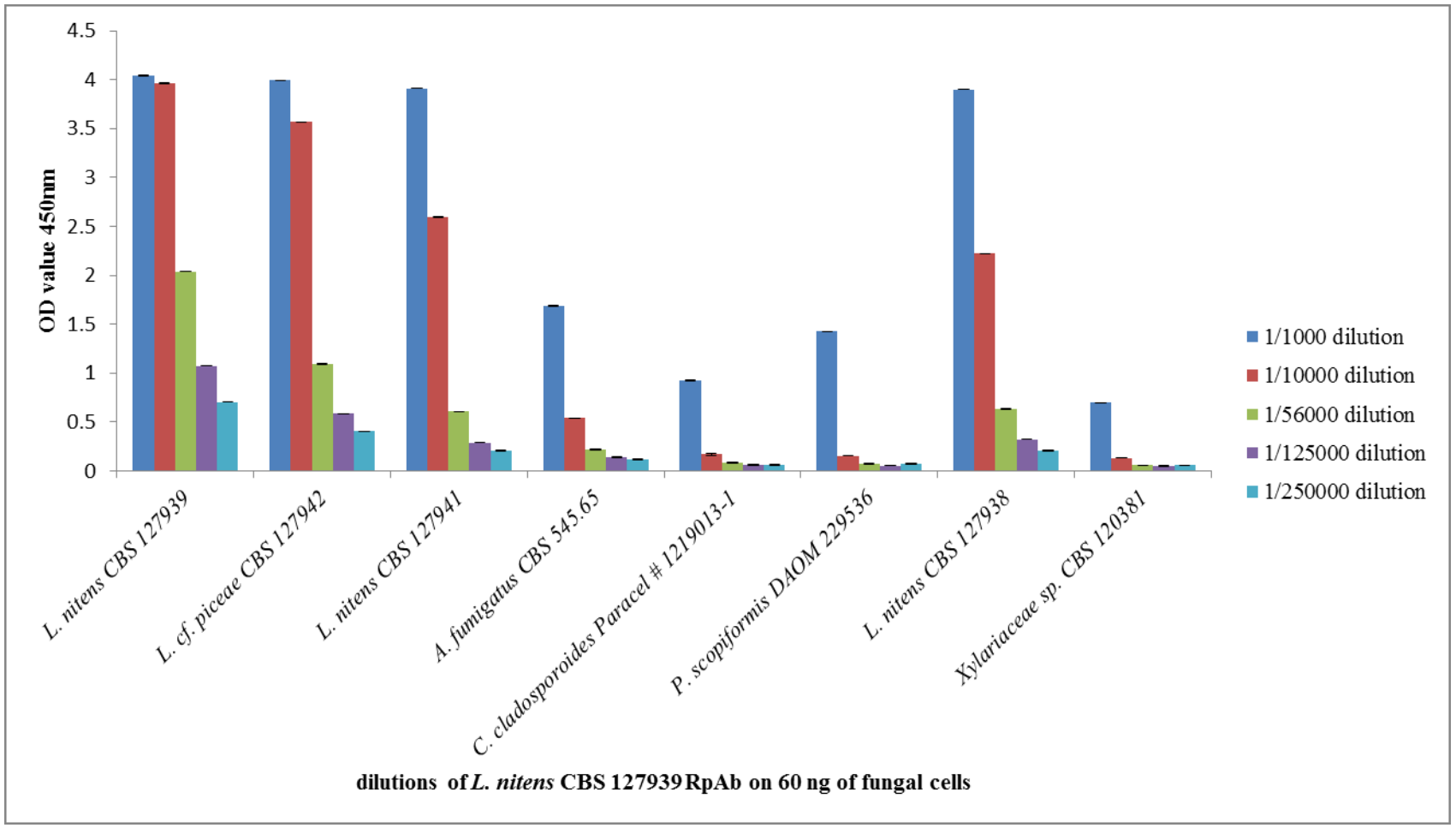

Figure 25. Cross reactivity of *Lophodermium nitens* CBS 127939 assay.

Plate coated with 60 ng of fungal cells from pure culture; blocking: 4hrs with Blotto; developing antibody: dilutions of *Lophodermium cf. piceae* CBS 127941 polyclonal antibody; detection: 1:10,000X HRPO. Data points represent an average from a portion of the same sample of mycelium done in 9 replicate microplate wells. Error bars represent standard error.

3.4.3 Needle testing

The effect of the needle matrix on tree samples was assessed by running assays with microplate wells coated with 500 ng of freeze dried, ground white pine needles and spiked with a range of target endophyte hyphae (30-100 ng). Quantification of the target endophytes were not affected at concentrations of 30 ng or 100 ng of cells by the presence of powdered freeze dried needles for any of the three assays (figure 26). Responses were evaluated using analysis of variance (ANOVA) Tukey's HSD test (*p* values < 0.001) and T-test: two sample assuming equal variance analysis. At 50 ng and 50 ng + 500 ng of needle in the *L. nitens* 127941 assay, a slight variance was seen (p= 0.047). This is near the limit of quantification for this assay in the needle matrix.

The experiments done did not determine the limit of detection i.e. the lowest value tested remained statistically significant from the blanks (ANOVA, followed by Tukey HSD). For this reason, the LOQ was estimated as the second lowest value, 50 ng mycelium for each case. Absorbance values were highly correlated indicating the linearity of the assay was not affected by the presence of the needle matrix. ANOVA with Tukey's HSD tested the response for 500 ng of needle material with 30 ng of target endophyte cells was significantly greater than that of the blanks ($p<0.001$) for each assay. For each assay, 50 ng of target endophyte was determined to be the LOQ in the presence of needle material.

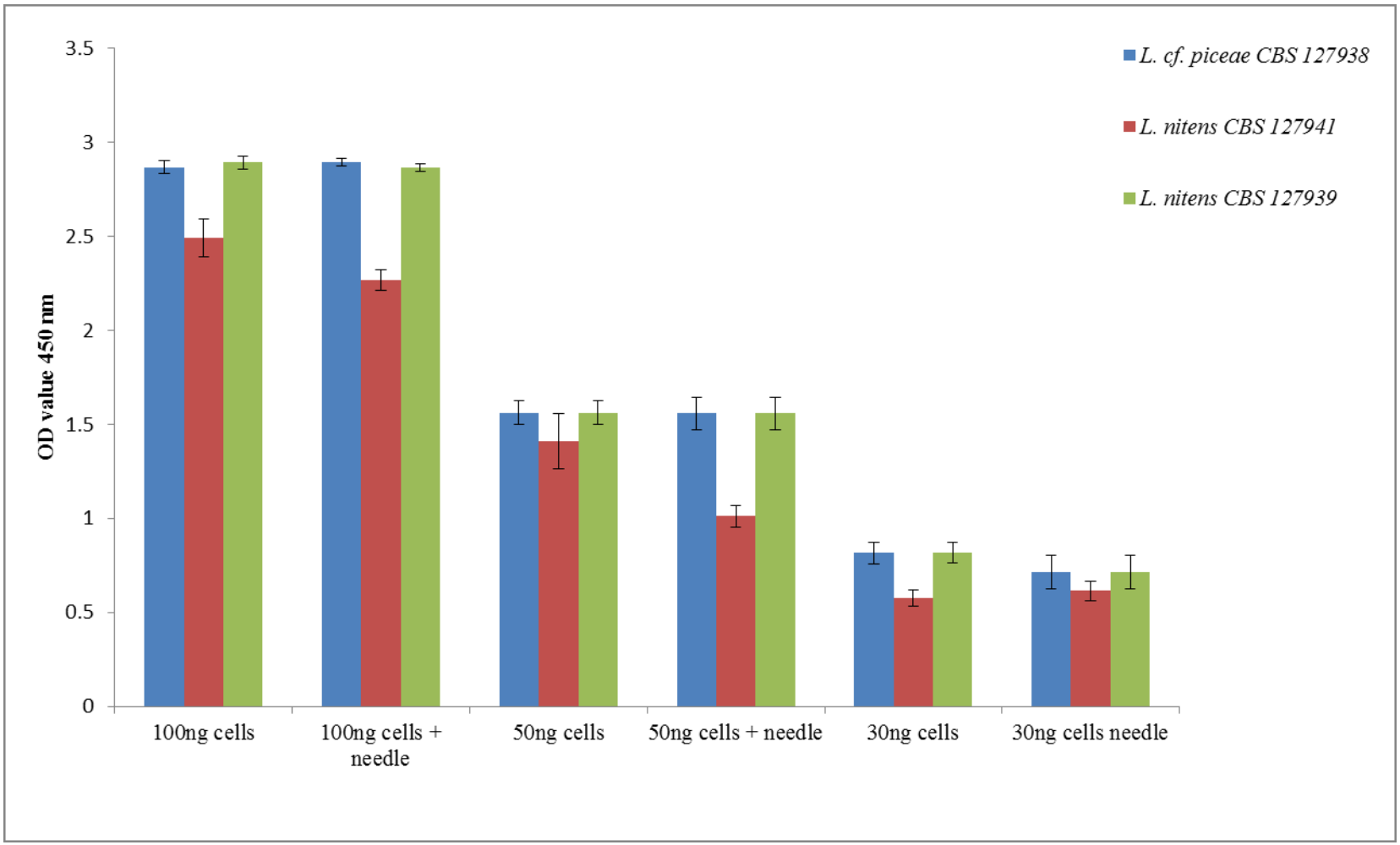

Figure 26. Indirect ELISA of 500ng of white pine needles spiked with target endophyte cells.

Plate coated with fungal cells from pure culture; blocking: 4hrs with Blotto; developing antibody: 1:56,000X dilutions of polyclonal antibodies; detection: 1:10,000X HRPO. Data points represent an average from a portion of the same sample of mycelium done in 9 replicate microplate wells. Error bars represent standard error.

3.4.4 Test samples

Needle samples of white pine inoculated with the target endophytes were tested using the optimized assays. For each tree sample, cells were diluted in coating buffer and tested in duplicate wells at concentrations of 100 ng/well and 500 ng/well. Needle samples of white pine inoculated with the endophyte *L. nitens* CBS 127941 as seedlings were tested using the ELISA method and 7.7% of the 52 samples tested showed a positive signal (table 2). Needle samples of white pine inoculated with the endophyte *L. nitens* CBS 127939 as seedlings were tested using the ELISA method and 7.0% of the 100 samples showed a positive signal (table 3).

Table 2. Needle samples of white pine inoculated with *Lophodermium nitens* CBS 127941 and tested with developed ELISA.

sample	ELISA +/-	sample	ELISA +/-	sample	ELISA +/-	sample	ELISA +/-
1	-	14	+	27	-	40	-
2	-	15	-	28	-	41	-
3	-	16	-	29	-	42	-
4	-	17	-	30	-	43	-
5	-	18	-	31	+	44	-
6	-	19	-	32	-	45	-
7	-	20	-	33	-	46	-
8	-	21	-	34	-	47	-
9	-	22	+	35	-	48	-
10	-	23	-	36	-	49	-
11	-	24	+	37	-	50	-
12	-	25	-	38	-	51	-
13	-	26	-	39	-	52	-

Table 3. Needle samples of white pine inoculated with *Lophodermium nitens* CBS 127939 and tested with developed ELISA.

sample	ELISA +/-	sample	ELISA +/-	sample	ELISA +/-	sample	ELISA +/-
1	-	26	-	51	-	76	-
2	-	27	+	52	-	77	-
3	-	28	-	53	-	78	-
4	-	29	-	54	-	79	-
5	-	30	+	55	-	80	-
6	-	31	-	56	-	81	-
7	-	32	-	57	-	82	-
8	-	33	-	58	-	83	+
9	-	34	-	59	-	84	-
10	-	35	-	60	-	85	-
11	-	36	+	61	-	86	-
12	-	37	-	62	-	87	-
13	+	38	-	63	-	88	-
14	-	39	-	64	-	89	-
15	-	40	-	65	-	90	-
16	-	41	-	66	-	91	-
17	+	42	-	67	-	92	-
18	-	43	-	68	-	93	-
19	-	44	-	69	-	94	-
20	-	45	-	70	-	95	-
21	-	46	-	71	-	96	-
22	-	47	-	72	-	97	-
23	+	48	-	73	-	98	-
24	-	49	-	74	-	99	-
25	-	50	-	75	-	100	-

3.5 Primer and TaqMan hydrolysis probe development

Quantitative PCR primers and TaqMan probes were designed using the alignment of relevant ITS sequences. The product length for the *P. scopiformis* DAOM 229536 assay was 91 base pairs long and product length for the *L. nitens* CBS 127941/*L. nitens* CBS 127939 assay was 149 base pairs long (table 4). Probes were labeled at the 5' end with the fluorescent reporter dye 6-carboxyflourescein (FAM) with the quencher dye 6-carboxy-tetramethylrhodamine (TAMRA) at the 3' end. Polymorphic regions (figure 27 and figure 28) that probes were designed around were one and two base pairs.

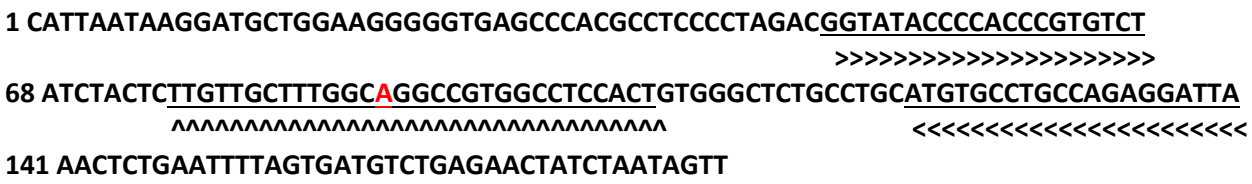

Figure 27. Target amplicon region of *Phialocephala scopiformis* DAOM 229536.

Forward primer (>), reverse primer (<), and probe (^) designed around nucleotide polymorphism (red) from ITS sequence alignment of phylloplane fungi and closely related endophyte species.

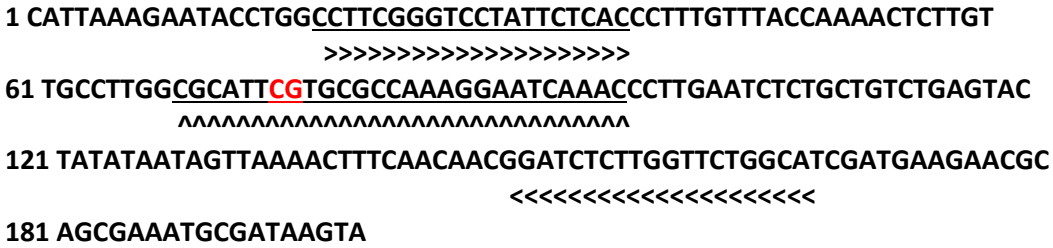

Figure 28. Target amplicon region of *Lophodermium nitens* CBS 127939/*Lophodermium nitens* CBS 127941.

Forward primer (>), reverse primer (<), and probe (^) designed around nucleotide polymorphism (red) from ITS sequence alignment of phylloplane fungi and closely related endophyte species.

Table 4. Primers and probe sequences for qPCR assays.

P. scopiformis DAOM 229536	
forward primer	5'-GGTATACCCCACCCGTGTCT-3'
reverse primer	5'-TAATCCTCTGGCAGGCACAT-3'
probe	5'-TTGTTGCTTTGGCAGGCCGTGGCCTCCACT-3'
L. nitens CBS 127941	
forward primer	5'-CCTTCGGGTCCTATTCTCAC-3'
reverse primer	5'-ATGCCAGAACCAAGAGATCC-3'
probe	5'-CGCATTCGTGCGCCAAAGGAATCAAAC-3'
L. nitens CBS 127939	
forward primer	5'-CCTTCGGGTCCTATTCTCAC-3'
reverse primer	5'-ATGCCAGAACCAAGAGATCC-3'
probe	5'-CGCATTCGTGCGCCAAAGGAATCAAAC-3'

3.6 PCR amplification

Tenfold serial dilutions ranging from 8.6 ng-8.6 fg for *P. scopiformis* DAOM 229536, 6.1 ng-6.1 fg *L. nitens* CBS 127941 and 3.1 ng-3.1 fg *L. nitens* CBS 127939 of DNA extracted from culture samples were used as templates for generating internal standard curves. Efficiency values ranged from 1.897 to 2.002 for all three assays with an average of 1.903 for the *P. scopiformis* DAOM 229536 assay, 1.997 for the *L. nitens* CBS 127941 assay and 1.966 for the *L. nitens* CBS 127939 assay. The assays were linear over 4, 5 and 5 logs respectively with R^2 values of 0.999, 0.997 and 0.993. Limits of detections were calculated to be 8.6^{-15} g DNA, 6.1^{-16} g DNA and 3.1^{-16} g DNA by extinction of fluorescent signal (figures 29-31), translating to 1 ng, 0.1 ng and 0.1 ng of mycelium.

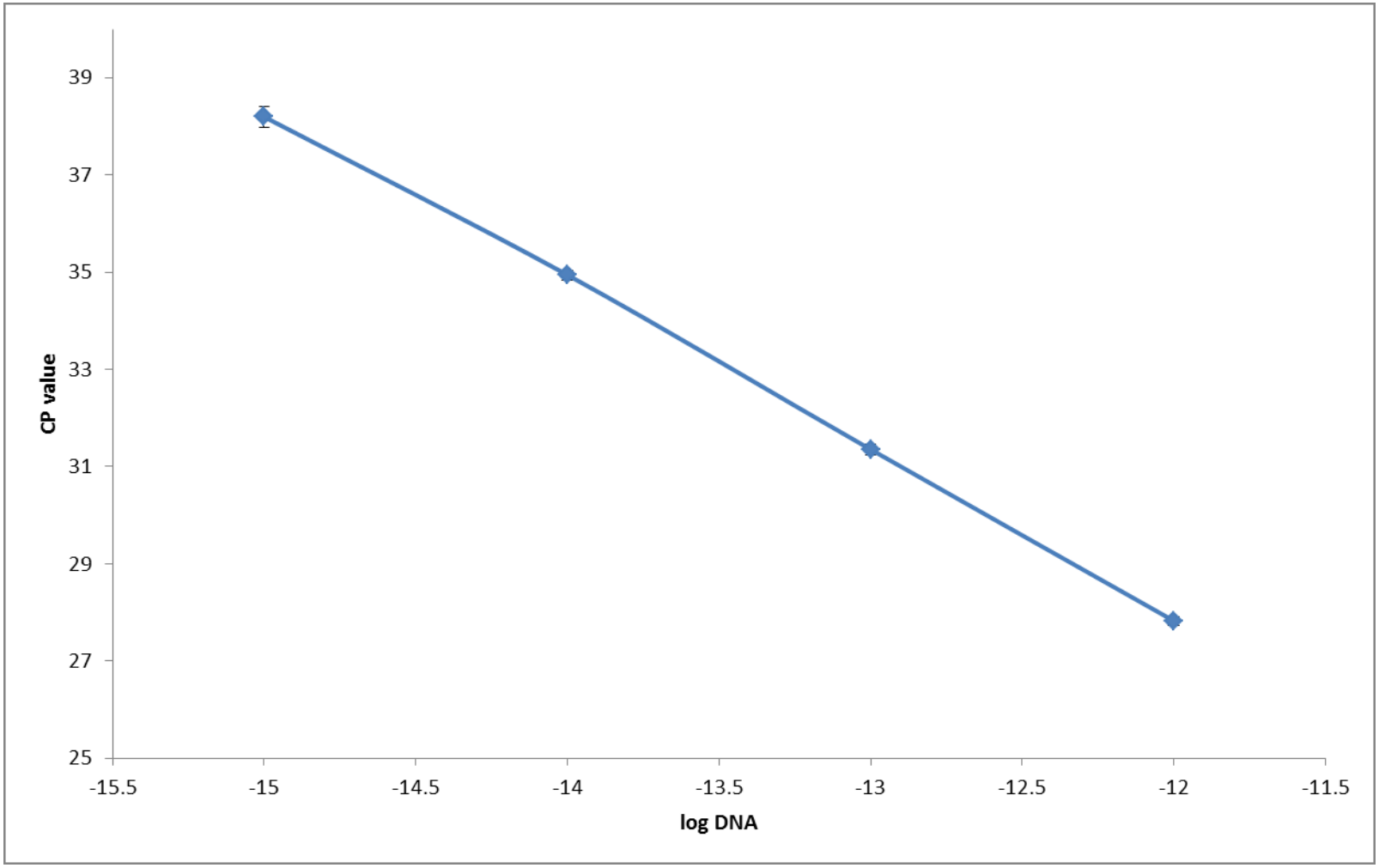

Figure 29. Standard curve of *Phialocephala scopiformis* DAOM 229536 qPCR assay.

Template DNA diluted from 8.6 x 10^-12 g to 8.6 x 10 ^-15 g DNA in ddH_20. Data points represent an average from a portion of the same sample of mycelium done in 24 replicate microplate wells. Where not visible, standard error bars are within the data point symbols. $R^2 = 0.9996$. Ef= 1.903 over 50 amplification cycles.

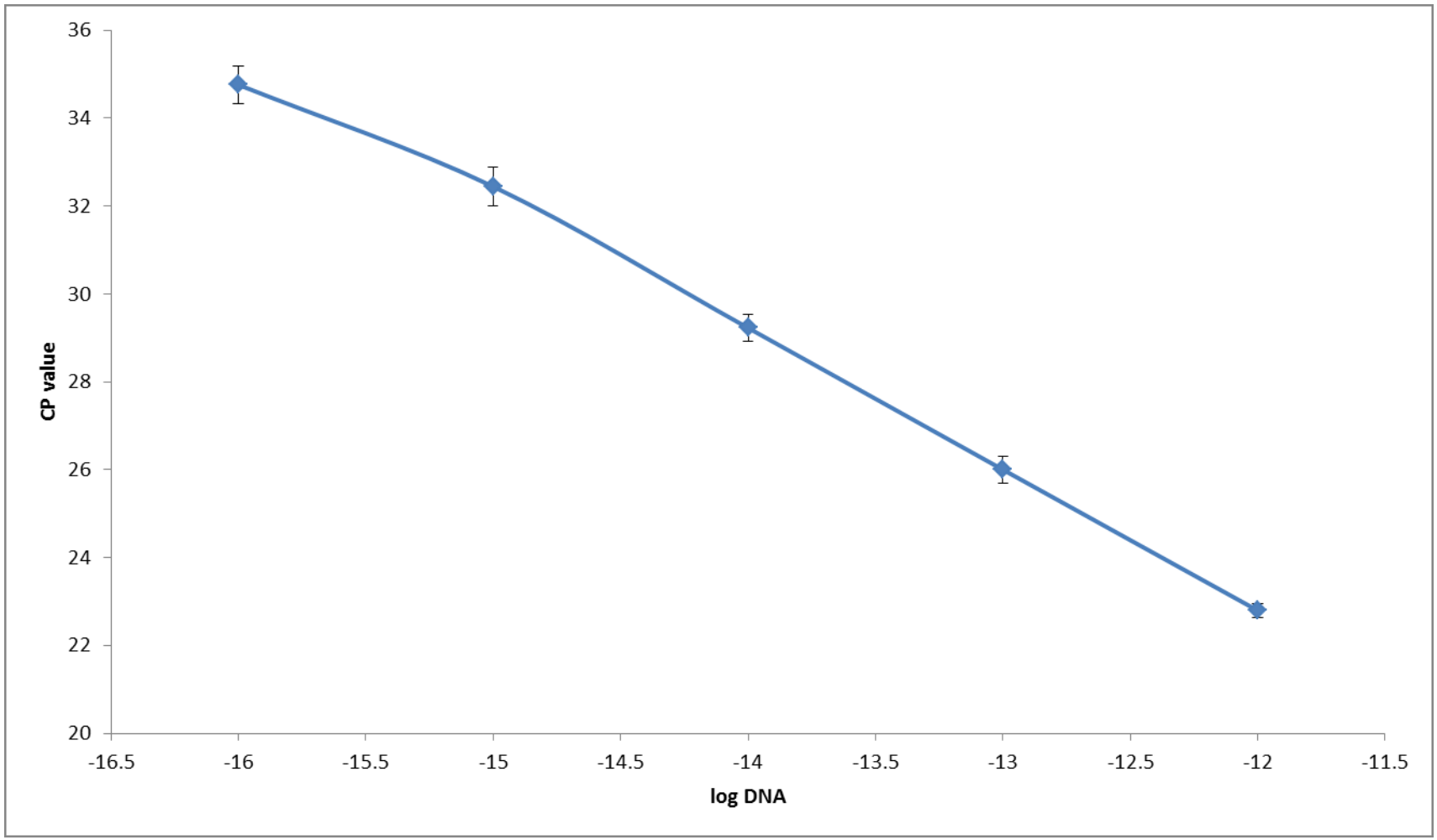

Figure 30. Standard curve of *Lophodermium nitens* CBS 127941.

Template DNA diluted from 6.1 x 10^-12 g to 6.1 x 10 ^-16 g DNA in ddH_20. Data points represent an average from a portion of the same sample of mycelium done in 9 replicate microplate wells. Where not visible, standard error bars are within the data point symbols. R^2 = 0.997. Ef= 1.997 over 50 amplification cycles.

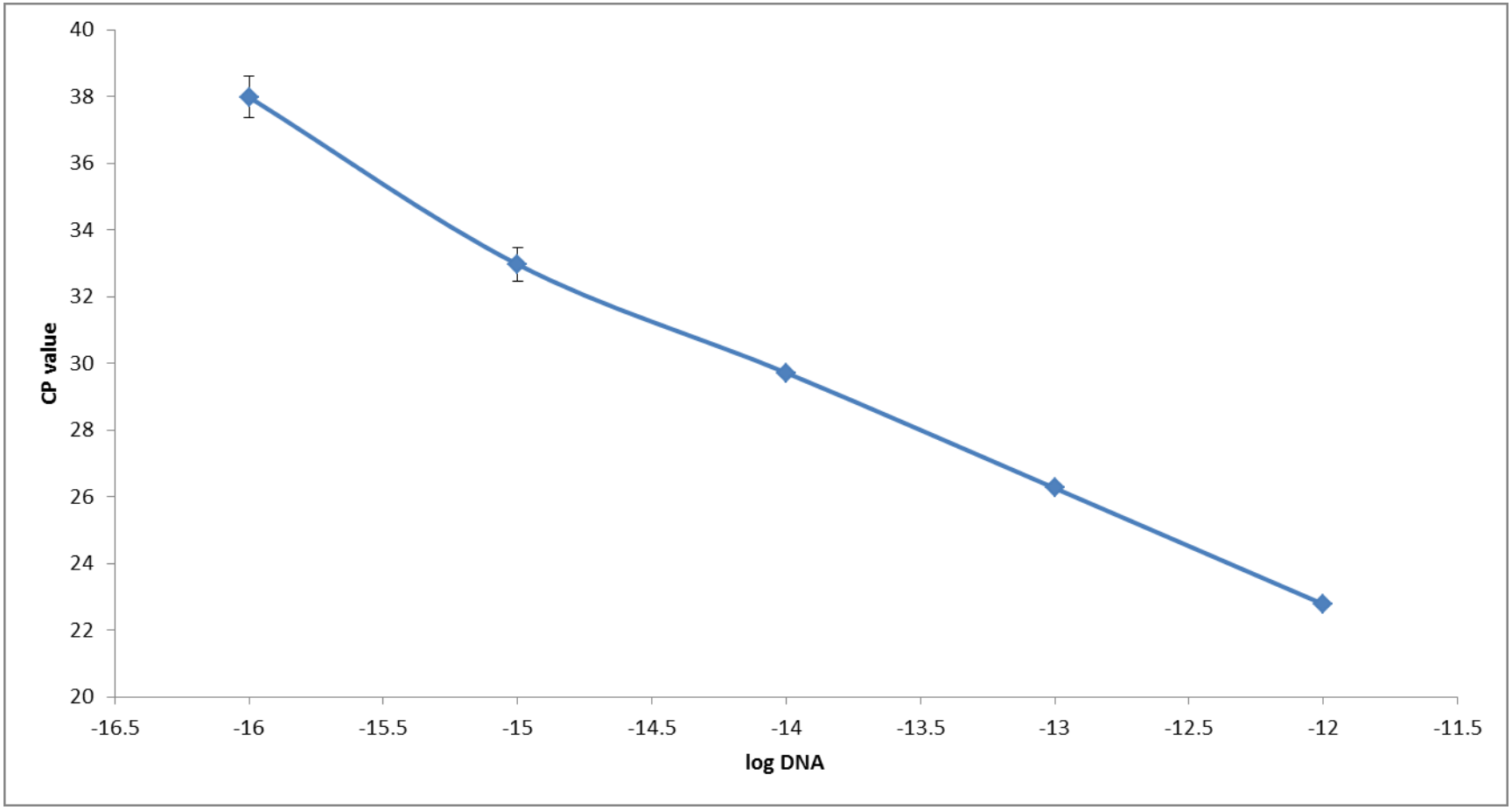

Figure 31. Standard curve of *Lophodermium nitens* CBS 127939.

Template DNA diluted from 3.1 x 10^-12 g to 3.1 x 10 ^-16 g DNA in ddH_20. Data points represent an average from a portion of the same sample of mycelium done in 9 replicate microplate wells. Where not visible, standard error bars are within the data point symbols. R^2 0.993. Ef= 1.966 over 50 amplification cycles.

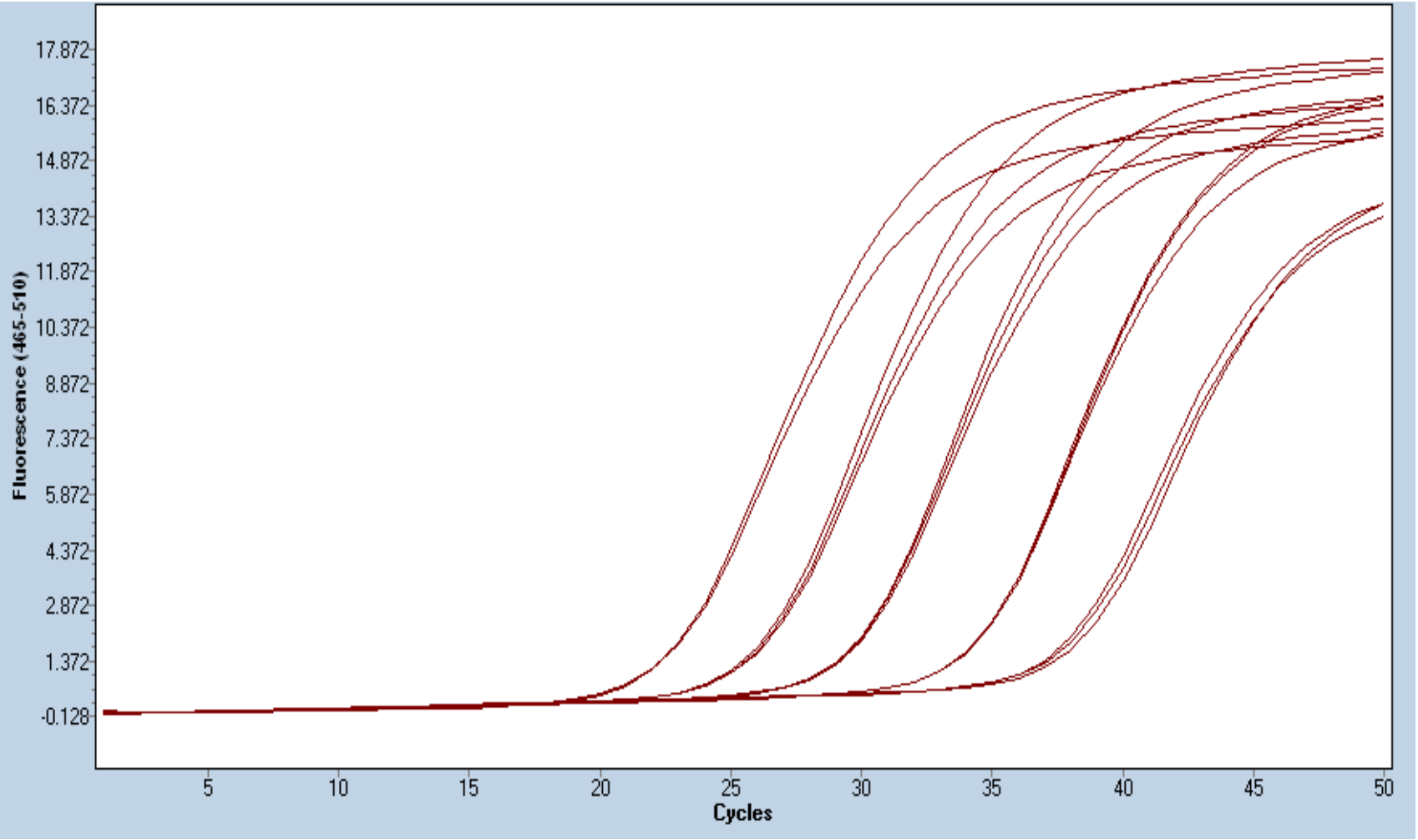

Figure 32. Representative internal standard curve of *Lophodermium nitens* CBS 127939 DNA.

Lophodermium nitens CBS 127939 DNA diluted from 3.1 x 10^$^{-12}$ g to 3.1 x 10 ^$^{-16}$ g DNA in dd$H_2$0.

3.7 Specificity testing

Cross reactivity testing showed the assays to be specific to the species and strains they were developed to detect (table 5). The specificity of the assay was investigated using cultures of common conifer foliar endophytes and phylloplane fungi collected from the same region. No signal was detected from two closely related *Lophodermium* strains or three *Phialocephala* species that were tested with the assay. An unknown *Phialocephala* isolated from the region of study did produce a positive qPCR signal but was subsequently sequenced and confirmed as *P. scopiformis* using the ITS gene region. Species of surface contaminants that occur on needles such as *Aspergillis, Cladosporium* and *Penicillium* (Hyde, 2008) were tested with the primer and TaqMan probes to confirm there was no cross reactivity *in vivo*.

Table 5. Cross reactivity against the developed probes *in vivo*.

species/strain	reactivity to *P. scopiformis* DAOM 229536 probe	reactivity to *Lophodermium nitens* CBS 127939/*Lophodermium nitens* CBS 127941
L. nitens CBS 127941	-	+
L. nitens CBS 127939	-	+
L. nitens CBS 127938	-	-
L. cf. piceae CBS 127942	-	-
P. scopiformis DAOM 229536	+	-
P. compacta CBS 507.94	-	-
P. dimorphospora CBS 300.12	-	-
P. fortinii FJ031031	-	-
A. fumigatus CBS 545.65	-	-
C. cladosporioides Paracel ID 1219013-1	-	-
P. chrysogenum DAOM 234056	-	-

A 3-log decrease was seen in the sensitivity of the *P. scopiformis* DAOM 229536 assay in the presence of a white spruce matrix (table 6). The method limit of quantification was 100 ng of mycelium /g of needle.

Table 6. Target DNA detected with *Phialocephala scopiformis* DAOM 229536 rt-PCR assay.

mycelium (mg)	*P. scopiformis* DAOM 229536 DNA (g)	*P. scopiformis* DAOM 229536 DNA from mycelium + 100mg needle
100	$1.10x10^{-9}$	$1.11x10^{-12}$
10	$2.12x10^{-11}$	$1.44x10^{-14}$
1	$1.90x10^{-12}$	$1.97x10^{-15}$
0.1	$8.94x10^{-13}$	0

3.8 Test samples

The P. scopiformis DAOM 229536 probe assay was tested on tree samples of a 13 year old white spruce tree confirmed to be infected with *P. scopiformis* DAOM 229536 by the ELISA of Sumarah et al. (2005). Samples also had previously been analysed for the *P. scopiformis* metabolite rugulosin after Miller et al. (2009). Briefly, an aliquot of 200 mg of freeze-dried ground needles was defatted using 20 mL petroleum ether for 50 min on ice. After filtration by suction, the petroleum ether extract was discarded and the suspension was extracted with chloroform (20 mL x 2, for 80 min each). The chloroform extract (10 mL) was washed with 10 mL of 5% NaHCO3 solution and the organic layer was discarded. The aqueous layer was

adjusted to pH 3 by the addition of 1 M HCl. The acidic solution was extracted with chloroform (10 mL x 2) and the organic layer was evaporated to dryness under a stream of nitrogen. The resulting extract was dissolved in 50 µL of MeOH and analyzed by HPLC. The LOQ for rugulosin was 150 ng/g. Out of the 109 samples tested with this assay, 100% of the rugulosin positive samples also tested positive for *P. scopiformis* DAOM 229536 DNA using our assay. A small number of samples (4) had tested negative for rugulosin but tested positive *for P. scopiformis* DAOM 229536 DNA and 13 samples negative for rugulosin were also negative for *P. scopiformis* DAOM 229536 DNA (table 7).

To determine the efficiency and reproducibility, a subset of 1200 samples inoculated with *P. scopiformis* DAOM 229536 were analyzed by the qPCR assay developed in the work. *P. scopiformmis* DAOM 229536 DNA was detected in 164 out of 291 samples (56.4%; table 8).

A subset of samples of 400 white pine seedlings inoculated with *L. nitens* CBS 127939 in a research nursery were developed using the developed assay. From samples tested, 38 out of the 100 samples (38%) were positive *for L. nitens* CBS 127939 DNA (table 10). Similarly, needle samples of white pine seedlings inoculated with *L. nitens* CBS 127941 were tested using the developed assay. From samples tested, 30 out of the 52 (58%) were positive for *L. nitens* CBS 127941 DNA (table 11).

Table 7. Rugulosin and *Phialocephala scopiformis* DAOM 229536 DNA in needle samples from a white spruce tree.

sample #	rug +/-	qPCR +/-	sample #	rug +/-	qPCR +/-	sample #	rug +/-	qPCR +/-	sample #	rug +/-	qPCR +/-	sample #	rug +/-	qPCR +/-
1043	+	+	843	+	+	643	+	+	443	+	+	243	+	+
1042	+	NES	842	+	+	642	+	+	442	-	+	242	+	NES
1041	+	+	841	+	+	641	+	+	441	-	+	241	+	+
1033	+	+	833	+	+	633	+	+	433	+	+	233	+	+
1032	+	+	832	+	+	632	+	+	432	+	+	232	+	+
1031	+	+	831	+	+	631	+	+	431	-	-	231	+	+
1023	+	NES	823	+	+	623	+	NES	423	+	+	223	-	+
1022	+	+	822	+	+	622	-	-	422	+	+	222	+	+
1021	+	+	821	+	+	621	-	-	421	+	+	221	+	+
1013	+	+	813	+	+	613	+	+	413	+	+	213	+	+
1012	+	+	812	+	+	612	+	+	412	+	+	212	+	+
1011	-	-	811	-	-	611	+	+	411	+	+	211	+	+
943	+	+	743	+	+	543	+	NES	343	+	+	142	-	-
942	+	+	742	+	+	542	+	+	342	+	+	141	+	+
941	+	+	741	-	-	541	-	-	341	-	-	132	+	+
933	+	+	733	+	+	533	-	-	333	+	+	131	+	+
932	+	+	732	+	+	532	+	+	332	-	-	122	+	+
931	+	+	731	+	+	531	+	+	331	+	+	121	+	+
923	+	+	723	+	+	523	+	+	323	+	NES	112	-	+
922	+	+	722	+	+	522	+	+	322	+	+	111	-	-
921	+	+	721	+	+	521	+	+	321	+	+			
913	+	+	713	+	+	513	-	-	313	+	NES			
912	-	-	712	+	+	512	+	+	312	+	+			
911	+	+	711	+	+	511	+	+	311	+	+			

White spruce tree samples were tested using the qPCR assay in triplicate wells. Positive (+) indicates at least one of three replicates were positive in 50 amplification cycles. Not enough sample (NES).

Table 8. *Phialocephala scopiformis* DAOM 229536 DNA in white spruce needle samples.

sample #	qPCR +/-	sample #	qPCR +/-	sample #	qPCR +/-	sample #	qPCR +/-	sample #	qPCR +/-	sample #	qPCR +/-	sample #	qPCR +/-
T11793	-	T11514	+	T11373	-	T11088	+	111970	-	111901	+	111595	-
T11748	-	T11511	-	T11340	-	T11082	+	111969	-	111896	-	111594	+
T11743	+	T11502	-	T11325	-	T11067	+	111967	+	111881	-	111593	+
T11703	+	T11499	+	T11319	+	T11064	+	111963	+	111712	+	111592	-
T11679	-	T11493	+	T11307	-	T11010	-	111961	-	111654	+	111591	+
T11673	+	T11490	+	T11304	+	T11009	-	111960	+	111648	+	111587	-
T11658	+	T11487	-	T11295	-	112002	+	111957	+	111646	-	111585	+
T11653	-	T11478	-	T11268	-	111992	+	111953	+	111638	-	111582	-
T11637	+	T11472	+	T11226	-	111991	+	111952	+	111632	+	111579	-
T11613	-	T11442	-	T11217	-	111988	+	111951	+	111625	+	111578	+
T11607	-	T11436	-	T11205	-	111986	-	111938	+	111624	+	111577	+
T11589	-	T11427	-	T11202	+	111982	-	111935	+	111619	+	111575	+
T11583	+	T11424	+	T11157	-	111979	-	111933	-	111611	-	111571	+
T11571	+	T11421	+	T11151	+	111977	-	111923	-	111605	-	111570	-
T11568	+	T11418	-	T11130	-	111973	-	111921	+	111602	+	111569	+
T11541	+	T11406	+	T11127	-	111972	+	111920	-	111597	+	111566	+
T11520	+	T11397	-	T11109	+	111971	-	111911	-	111596	-	111562	-
111223	-	111163	+	110886	+	110718	+	110608	+	110539	-	110284	+
111216	-	111161	-	110883	-	110705	+	110606	+	110536	-	110277	+
111215	+	111149	-	110876	+	110690	+	110599	-	110528	-	110274	+
111209	+	111148	-	110744	+	110686	+	110596	+	110527	+	110269	-
111206	+	111141	+	110741	-	110679	-	110595	-	110503	-	110263	+
111202	+	111136	+	110738	+	110668	-	110581	-	110501	+	110262	+
111198	-	111130	-	110734	+	110658	+	110577	-	110500	-	110254	-
111197	+	111126	+	110733	+	110654	+	110573	-	110499	-	110249	+
111192	-	111125	+	110732	+	110650	+	110569	-	110496	+	110249	+
111191	+	111107	+	110731	+	110627	-	110565	-	110305	+	110231	+

White spruce tree samples were tested using the qPCR assay in duplicate wells. Positive (+) indicates at least one of two replicates were positive in 50 amplification cycles.

Table 8 Continued. *Phialocephala scopiformis* DAOM 229536 DNA in white pine spruce samples.

sample #	qPCR +/-	sample #	qPCR +/-	sample #	qPCR +/-	sample #	qPCR +/-	sample #	qPCR +/-	sample #	qPCR +/-	sample #	qPCR +/-
111190	-	110931	+	110730	+	110626	+	110562	+	110302	+	110226	-
111189	-	110924	+	110729	-	110624	-	110557	+	110295	+	110225	-
111185	+	110916	-	110728	-	110623	+	110556	+	110292	-	110222	-
111183	+	110905	+	110727	-	110612	+	110548	+	110290	+	110221	-
111180	-	110899	+	110726	+	110611	+	110545	-	110289	+	110219	-
111178	-	110897	-	110721	+	110610	+	110009	-	110013	-	110544	-
110014	-	110287	+	110026	+	110211	-	110027	+	100901	-	110028	+
110030	+	110208	+	110061	+	111168	+	100901	-	111229	+	110067	+
110044	+	110609	-	110059	+	110893	+	110063	+	100742	+	111231	-
110037	-	110543	+	110046	+	110719	-	110062	+	100631	+	110070	+
110034	-	110285	+	111226	-	110064	+	111227	-	110065	-	110099	-
111548	-	110096	+	111560	-	110133	-	111562	-	110207	+	111551	+
111542	+	110094	-	111557	+	110117	-	111561	-	110201	+	111550	+
111536	+	110091	+	111556	-	110113	+	111232	+	110077	+	110100	+
111393	-	110079	+	111555	+	110102	+						

White spruce tree samples were tested using the qPCR assay in duplicate wells. Positive (+) indicates at least one of two replicates were positive in 50 amplification cycles.

Table 9. Repeat testing for *Phialocephala scopiformis* DAOM 229536 DNA in white spruce needle samples.

sample #	qPCR +/-	qPCR +/-	sample #	qPCR +/-	qPCR +/-	sample #	qPCR +/-	qPCR +/-
T11502	-	-	111594	+	+	110545	-	-
T11499	+	-	111593	+	+	110503	-	-
T11436	-	+	111587	-	-	110500	-	-
T11421	+	+	111570	-	-	110289	+	+
T11406	+	+	111550	+	-	110287	+	+
T11268	-	-	111542	+	+	110284	+	+
111988	+	-	111232	+	+	110274	+	+
111960	+	+	111227	-	-	110249	+	+
111957	+	+	111180	-	-	110226	-	+
111901	+	+	111141	+	-	110211	-	-
111654	+	+	110731	+	+	110208	+	+
111611	-	-	110730	+	+			

White spruce tree samples were tested using the qPCR assay in duplicate wells. Positive (+) indicates at least one of two replicates were positive in 50 amplification cycles.

Table 10. *Lophodermium nitens* CBS 127939 DNA in white pine needle samples.

sample #	qPCR +/-	sample #	qPCR +/-	sample #	qPCR +/-	sample #	qPCR +/-	sample #	qPCR +/-	sample #	qPCR +/-
1	-	18	-	35	-	52	-	69	+	86	-
2	-	19	-	36	-	53	-	70	-	87	-
3	-	20	-	37	-	54	+	71	+	88	-
4	-	21	-	38	-	55	-	72	-	89	+
5	-	22	+	39	+	56	+	73	+	90	-
6	+	23	-	40	-	57	-	74	-	91	-
7	+	24	+	41	+	58	+	75	-	92	-
8	-	25	+	42	-	59	-	76	-	93	-
9	+	26	-	43	+	60	+	77	-	94	-
10	-	27	-	44	-	61	-	78	+	95	-
11	+	28	-	45	-	62	-	79	-	96	+
12	+	29	-	46	-	63	+	80	+	97	+
13	+	30	-	47	+	64	+	81	+	98	-
14	+	31	+	48	+	65	-	82	-	99	+
15	-	32	-	49	-	66	-	83	-	100	+
16	-	33	-	50	+	67	+	84	-		
17	+	34	-	51	+	68	+	85	-		

White pine tree samples were tested using the qPCR assay in triplicate wells. Positive (+) indicates at least one of three replicates were positive in 50 amplification cycles.

Table 11. *Lophodermium nitens* CBS 127941 DNA in white pine needle samples.

sample #	qPCR +/-	sample #	qPCR +/-	sample #	qPCR +/-	sample #	qPCR +/-
1	+	14	-	27	+	40	-
2	-	15	-	28	+	41	-
3	+	16	+	29	+	42	+
4	-	17	+	30	+	43	+
5	+	18	+	31	-	44	+
6	-	19	+	32	+	45	+
7	-	20	-	33	+	46	-
8	-	21	-	34	-	47	+
9	+	22	-	35	-	48	-
10	+	23	-	36	+	49	+
11	+	24	-	37	+	50	+
12	-	25	+	38	+	51	+
13	+	26	-	39	+	52	-

White spruce tree samples were tested using the qPCR assay in triplicate wells. Positive (+) indicates at least one of three replicates were positive in 50 amplification cycles.

DISCUSSION

4.1 Biological controls

There are products available that are based on pathogenic fungi to control invertebrate pests, weeds and diseases and more products are being developed. Fungi to control parasites, nematodes and other fungi have been investigated (Butt & Copping 2000). Fungal biological control has implications for plant productivity as well as animal and human health (Butt et al. 2001). This field is a multi-disciplinary area of study requiring pathology, ecology, genetics and physiology.

It is generally recognized that some chemical pesticides contaminate ground water and pose hazards to animal health. Consumer perception is that the use of chemicals in agriculture should be reduced (Butt et al. 2001). There are two widely used types of biocontrols, classical biocontrol establishes an organism from an 'exotic' region to give permanent and long term pest control. The classical approach is typically done by a government organization for public interest. Inudative control is the periodic release of mass-produced natural enemies into specific environments to reduce pest populations. This type of method is usually marketed by industry, like a herbicide. Canada's first registered fungal biological control was the fungus *Colletotrichum gloeosporioides*. It met the Canadian commercial standard as an inudative biocontrol for the pest round-leaved mallow that is an in-crop weed problem (Harris 1993; Mason & Gillespie 2013). This bioherbicide was not commercialized due to a limited market size (Mason & Gillespie 2013).

In the search for commercially viable fungal biocontrol agents, possible successful agents must be isolated from the environment. Studies to understand the ecology and physiology of the organisms are important to understand the effect of environmental factors on the survival and distribution of the organisms. The application of inoculum and promotion of amplification of the agent are important considerations. Laboratory studies and field bioassays help to determine the most antagonistic and ecologically fit strains. Dose-mortality studies determine the amount of inoculum required to cause disease and supress plant pathogens. These studies also are indicative of the time it takes for an agent to have an impact on target organisms (Butt et al. 2001).

Endophytes are microorganisms that live within plants without causing any visible manifestation of disease (Bacon & White 2000). Endophytic fungi infect living plant tissue and typically remain with the healthy plant for the lifespan of their host. Endophytes are a diverse polyphyletic group of microorganisms, thriving asymptomatically in plant tissue such as stems leaves and roots (Kusari et al. 2012). Aside from the grass endophytes previously discussed, there are emerging examples of positive plant-endophyte associations. For example, Bittleston et al. (2010) showed that a species of defoliating leaf-cutting ant (*Atta colombica*) had a significant preference for seedlings with a low endophyte infection load. The maize endophyte *Acremonium zeae* is antagonistic to the mycotoxin producing fungi *Aspergillus flavus* and *Fusarium verticillioides* (Wicklow et al. 2005). Larkin et al. (2012) suggested that needle endophytes affect deer herbivory of Western white pine (*Pinus monticola*).

The successful inoculation of white spruce trees with *P. scopiformis*, a rugulosin-producing needle endophyte, reduced spruce budworm growth in chamber studies, under nursery conditions and in the field (Miller et al. 2002; Quiring et al. 2014; Sumarah et al. 2005; Sumarah et al. 2008). Inoculated seedlings maintained the endophyte when tested again 5 years later and rugulosin was detected in needles using high performance liquid chromatography (HPLC) (Sumarah et al. 2005). Miller et al. (2008) showed that seedlings can be inoculated with a beneficial foliar endophyte, the endophyte is maintained in the trees and the endophyte continues to produce a toxin that slows the growth of a serious insect pest. In a series of studies, (Miller et al. 2002, 2008; Sumarah et al. 2008, 2008; Sumarah & Miller 2009) it was demonstrated that certain endophytes and their metabolites provide the trees with a tolerance against the spruce budworm and can be incorporated under tree production conditions (Miller 2008). In successive experiments in the same test plot (Millet et al. 2002; Sumarah et al. 2005) it was observed that inoculated fungal endpohytes grow and colonize a tree slowly. When tested by ELISA 15 months post inoculation, 90% of seedlings were positive for the endophyte, a 75% increase when tested for the endophyte at 3 months post inoculation.

The use of toxigenic foliar endophytes for forestation and seedling stock has the potential for improving tolerance in various conifer species (Miller 2011). This would have long-lasting effects and increase the fitness of a tree stand (Miller 2011). For over 25 years, research has been done to determine how many populations of toxigenic endophytic fungal genotypes are in the Acadian forest (Miller 2011). Representative cultures have been inoculated back into seedlings used in reforestation attempts, restoring relevant genotypes at a tree stand level, avoiding monoculturing (Sumarah & Miller 2009) which can lead to a quicker spread of pests and disease.

A large-scale planting of endophyte infected fescue grass has successfully been implemented on golf courses and lawns (Charbonneau 1997) as a way to reduce the use of pesticide (Health Canada 2000). New cultivars produced through plant breeding are deliberately infected with fescue endophytes and sold to the public. Using a similar model with foliar endophytes, the inoculation of commercially important conifers will bestow important benefits to trees against harmful insect pests such as the spruce budworm (Kuldau & Bacon 2008; Miller 2011).

4.2 Immunoassays

Methods to confirm the successful inoculation of seedlings are critical for understanding the ecology and applicability of using foliar endophytes in commerce in conifers. Foliar endophytes of conifers like white spruce and white pine are transmitted horizontally, unlike the vertical transmission of grass endophytes (Sumarah et al. 2009; Miller et al. 2009).

To develop ELISA methods for foliar endophytes, extra-cellular proteins released by fungi were harvested. The temperature, growth period and nutrient content of cultures were strictly controlled. Growth of the fungi in 2% ME medium produced a similar antigen profile as the use of endophyte mycelium produce on irradiated spruce needles (Sumarah 2008). The extraction of proteins using a concentrator with a cut-off membrane has shown to be effective for the isolation of fungal antigens (Provost et al. 2013; Desroches et al. 2014). In this study, crude protein was isolated from 2% ME culture filtrate and concentrated using a concentrator with a 10,000 Da membrane. Crude protein was isolated from filtrate extracts of *L. nitens* CBS 127938, *L. nitens* CBS 127939, *L. nitens* CBS 127941 and *L. cf. piceae* CBS 127942 (figures 10-14).

Rabbit polyclonal antibodies (RpAb) were produced by immunizing rabbits with protein extract from *L. nitens* CBS 127939, *L. nitens* CBS 127941 and *L. cf. piceae* CBS 127942. Activity of the RpAbs was verified by indirect ELISA with the purified antigen and high responses were observed with both rabbit test bleeds and final bleeds. *L. nitens* CBS 127938 did not proceed to RpAb production because of progress made with qPCR detection methods.

Varying antibody and antigen concentrations were tested to optimize the indirect ELISA detection signal (OD value). Coating a microplate with excess antigen can cause passive binding and increase background. Applying excess primary antibody can result in non-specific binding. A weak signal will be obtained if the plate is coated with too little antigen because of the low epitope (the part of the antigen the antibody recognizes) density. If over-diluted, the primary antibody will fail to detect the epitopes that are present. Optimal amounts of coating antigen in the form of cells from the target endophyte and primary antibody were identified to maximize the detection signal while maintaining a minimal background. The estimated LOD for *L. cf. piceae* CBS 127942 assay was 30 ng of cells ($p<0.001$). The LOD for *L. nitens* CBS 127941 assay was 30 ng of cells ($p<0.001$). The LOD for *L. nitens* CBS 127939 was 30 ng of cells ($p<0.001$). The LOD for the optimized assays are comparable to the Sumarah et al. (2005) study detecting the presence of *P. scopiformis* in needle samples where the LOD was also 30 ng. Limit of quantification (LOQ) was determined to be 50 ng of target endophyte in the presence of needle material for the *L. cf. piceae* CBS 127942, *L. nitens* CBS 127941 and *L. nitens* CBS 127939 assays.

These assays were developed to detect the presence of the specific endophytes in conifer tree samples so the effects that conifer needles could have on the assay were assessed. Microplate wells were coated with 500 ng of freeze dried ground white pine needles and spiked with target endophyte cells. Quantifications of the target endophytes were not affected by the presence of powdered freeze dried needles except for the *L. nitens* 127941 assay at the limit of quantification (figure 26).

Each polyclonal antibody was tested for cross reactivity against *A. fumigatus* CBS 545.65, *C. cladosporioides* Paracel ID 1219013-1, *P. scopiformis* DAOM 229536, *L. nitens* CBS 127938 and a strain in the Xylariaceae CBS 120381. *A. fumigatus* is an insect pathogen commonly found on budworm associated conifers (Miller et al. 1984; Miller et al. 1985; Sumarah et al. 2008). Relative cross reactivity to the phylloplane fungi and *P. scopiformis* was minimal across antibody and cell dilutions for each assay. Greater cross reactivity was observed between the *Lophodermium* strains. This was not unexpected because these are closely related. The ELISAs in this study were produced to detect specific endophytes in seedlings inoculated with target species. The likelihood of seedlings being infected with a non-target endophyte in a nursery is very small due to horizontal transmission (Miller et al. 2002), allowing for the practical use of these assays.

Needle samples of white pine inoculated with the endophyte *L. nitens* CBS 127941 as seedlings were tested using the ELISA method and 7.7% of the 52 samples tested showed a positive signal

(table 2). Needle samples of white pine inoculated with the endophyte *L. nitens* CBS 127939 as seedlings were tested using the ELISA method and 7.0% of the 100 samples showed a positive signal (table 3). No needle samples inoculated with *L. cf. piceae* CBS 127942 were available at the time of testing. This is the first time inoculated foliar endophytes were successfully detected in white pine trees, an important step towards the viability of specific foliar endophytes in the forestry industry.

4.3 qPCR assays

Studies detecting and identifying fungi from environmental samples using qPCR are numerous throughout the literature (Cullen et al. 2001; Winton et al. 2002; Hietala et al. 2003; Bilodeau et al. 2006; Hogg et al. 2007; Demeke et al. 2010; Zhao et al. 2012). qPCR is highly sensitive and specific for the detection of nucleic acids (Hogg et al. 2007). It has become a popular diagnostic tool for the rapid and sensitive detection of pathogens (Winton et al. 2002; Hogg et al. 2007; Zhao et al. 2012).

Compared to conventional PCR, qPCR has significant advantages. With qPCR there is no need for any post-amplification process such as gels or sequencing, greatly reducing the time and labour required. High throughput systems are now available for large-scale analysis, increasing the practicality of doing these assays (Schena et al. 2002; Crossley et al. 2003). Some studies have demonstrated qPCR to be more sensitive than conventional PCR. Fungi were detected in soil and plant material using qPCR but not as often when using conventional PCR (Lees et al. 2002; Cullen et al. 2001). Other studies claim that conventional PCR is preferable (Hierl et al.

2004; Hafez et al. 2005). The reduced sample manipulation with qPCR compared to conventional PCR is suggested to reduce potential cross contamination from reaction mixtures or carry-over DNA (Schena et al. 2004).

There are naturally occurring compounds such as humic acids, tannins and lignin associated compounds that interfere with PCR reactions and inhibit amplification (Cullen and Hirsch 1998; Bridge and Spooner 2001; Ippolito et al. 2002; Keswani et al 2005). These issues have been partially addressed by the optimization of DNA isolation methods. qPCR is less affected by these inhibitors because they affect the late cycles of PCR, during the product accumulation (Mumford et al. 2000). With qPCR, product accumulation is not required to give a positive result; detection is achieved through the generation of a fluorescent signal in the early stages (Mumford et al. 2000).

The ability to design PCR primers and probes to target specific regions of DNA has led to a greater understanding of fungal ecology, plant-fungi interactions and fungal-fungal interactions (Atkins & Clark 2004). An eventual goal for qPCR would be to develop primer and probe based detection kits that could be taken into the field to detect pathogens or other fungi of interest. Selecting target DNA using sequence information allows primers and probes to be designed around conserved and variable regions (Atkins & Clark 2004). Fungal nuclear ribosomal DNA consists of three genes, as discussed in the introduction. These three genes are separated by the internal transcribed spacer (ITS) regions. The ITS region has areas of conservation and

variability, an ideal starting place for the development of specific primers and probes; as well it is the formally acknowledged barcode region for fungal species recognition (Schoch et al. 2012).

In this study, primers and TaqMan probes were designed using the alignment of relevant ITS sequences from regionally associated fungi and the most closely related fungal species to the target species (figure 27, 28). Probes were centered on polymorphisms unique to the target ITS sequences. Assays have successfully been designed around single nucleotide polymorphisms (Mein et al. 2000; Prince et al. 2001). Primers and probes were designed for the detection of *P. scopiformis* DAOM 229536, *L. nitens* CBS 127941 and *L. nitens* CBS 127939. The secondary structures of amplicon regions were analysed using Geneious R6 software to ensure maximal hybridization of the probe to the amplicon region. During probe design, failure to hybridize is a common issue, often related to amplicon length and secondary structure (Lane et al. 2004).

Figures 29-33 show standard calibration curves generated during each assay. For *P. scopiformis* DAOM 229536, *L. nitens* CBS 127941 and *L. nitens* CBS 127939, the assays were linear over 4, 5 and 5 logs respectively with R^2 values of 0.999, 0.997 and 0.993. Limits of detections were calculated to be 8.6^{-15} g DNA, 6.1^{-16} g DNA and 3.1^{-16} g DNA by extinction of fluorescent signal, translating to 1 ng, 0.1 ng and 0.1 ng of mycelium.

Efficiency values ranged from 1.897 to 2.002 for all three assays with an average of 1.903 for the *P. scopiformis* DAOM 229536 assay, 1.997 for the *L. nitens* CBS 127941 assay and 1.966 for the

L. nitens CBS 127939 assay. Although it has been the convention to work with logarithmic dilutions, it is well understood that optimal compositions of PCR components in appropriate proportions to template DNA result in reliable efficiencies (Towe et al. 2010). These efficiency values are critical for analysis of qPCR data. Efficiency values are generated from linear regression lines calculated between Ct values and logarithmized concentrations of template DNA using $Eff_{slope}= [10^{(-1/slope)}-1]$ (Towe et al. 2010). Most analyses of data are performed automatically by the software on a qPCR apparatus, especially the calculation of the Ct (Ruijter et al. 2009). An improper baseline setting will severely affect estimated PCR efficiencies and variability in the quantification on DNA (Ruijter et al. 2009). A perfect amplification reaction will have an efficiency of 2.00, meaning that every PCR product is replicated once in every cycle.

Cross reactivity testing showed the assays to be specific to the strains they were developed to detect (table 5). Phylloplane and closely related fungi showed no positive signals during testing. Primers and probes can be re-assessed for cross reactivity using *in silico* alignment tests as newly sequenced species become available. The bank of conifer endophyte sequences is relatively small but efforts are being made to compile a more thorough collection.

A 3-log decrease in the sensitivity of the *P. scopiformis* DAOM 229536 assay was observed in the presence of a white spruce needle matrix. Competition between plant and fungal samples during DNA extraction as well as PCR inhibitors such as polyphenols, tannins and polysaccharides inhibit enzymatic action (Porebski et al. 1997). Polysaccharides found in plant

material directly inhibit *Taq* polymerase activity (Fang et al. 1992). The *P. scopiformis* DAOM 229536 assay limit of detection in the presence of pine needles was 100 ng/g of needle. A 3-log decrease in sensitivity in the presence of needles can be extrapolated to the *L. nitens* CBS 127939 and *L. nitens* CBS 127941 assays, making the LOD on the presence of needles 10 ng/g of needle.

The *P. scopiformis* DAOM 229536 probe assay was tested on tree samples of a 13 year old white spruce tree confirmed to be infected with *P. scopiformis* DAOM 229536 with a previously developed ELISA. Samples had previously been analysed for the secondary metabolite rugulosin. Out of the 109 samples tested with this assay, 100% of the rugulosin positive samples also tested positive for *P. scopiformis* DAOM 229536 DNA. There were 4 samples that tested negative for rugulosin but tested positive for *P. scopiformis* DAOM 229536 DNA. And 13 samples negative for rugulosin were also negative for *P. scopiformis* DAOM 229536 DNA (table 7). It was found that the endophyte and its toxin were distributed throughout the crown of the tree. Approximately 90% of the samples taken from the crown were positive for rugulosin. The mean and median of positive samples contained 1.2 and 0.93 µg/g of rugulosin respectively, greater than twice the low adverse effect concentration for spruce budworm growth on needles (Miller 2011). There was 100% concordance between the qPCR and positive analytical data. There were 4 samples positive by qPCR but below the LOD for rugulosin.

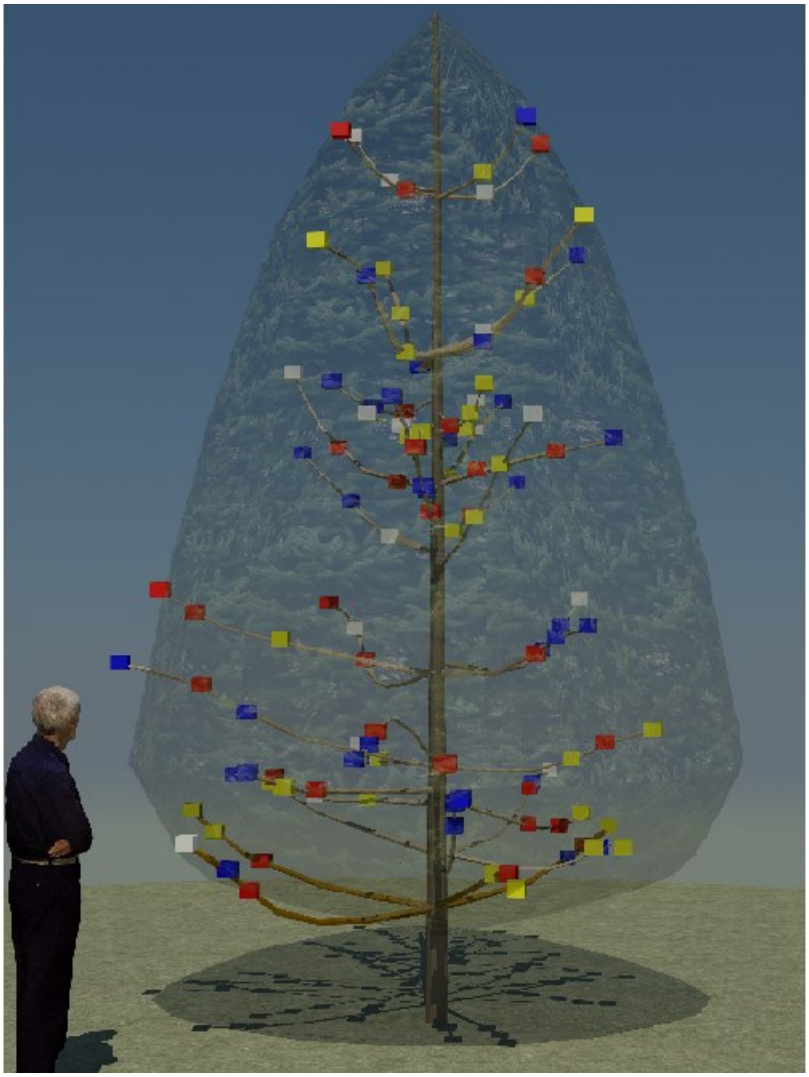

Figure 35. Rugulosin concentrations in needles as determined by HPLC.

Range of rugulosin concentrations throughout the crown of the test white spruce tree as determined by high performance liquid chromatography. (white= undetected, red= >0-0.69 µg/g, green= 0.7-1.39 µg/g, blue= >1.40 µg/g of needle). Figure courtesy of Wayne Handspiker (JD Irving).

A larger scale trial to test the utility and reproducibility validity of the assay was done on white spruce samples inoculated with *P. scopiformis* DAOM 229536 in a commercial nursery. *P.*

scopiformis DAOM 229536 DNA was detected in 164 out of 291 samples (56.4%; table 8). To determine the reproducibility of the assay, 35 samples (12%) were extracted and retested. From these retested samples, 29 of the 35 (83%) showed the same result as previously obtained (table 9). Needle samples of the white pine seedlings inoculated with *L. nitens* CBS 127939 under experimental conditions and previously tested by ELISA were tested using the qPCR assay. *L. nitens* CBS 127939 DNA was detected in 38 out of 100 samples tested (table 10). Needle samples of white pine seedlings inoculated with *L. nitens* CBS 127941 were tested using the qPCR assay. *L. nitens* CBS 127941 DNA in 30 out of the 52 tested (58%) were positive for (table 11).

4.4 Assay Comparison

It took over two months to grow the endophyte cultures to a point where enough extracellular protein was produced for extraction. Rabbit polyclonal antibody production requires over three months. The optimization of the ELISA method is labour intensive and testing samples is done over a two day period. The DNA isolation and sequencing for real-time PCR is done within a week and primers and probes can be designed and ordered in days. DNA can be isolated and analyzed with the assay the same day.

Limits of detection were 30 ng of pure culture for ELISA and 0.1-1.0 ng of pure culture for qPCR. Limits of quantification were 50 ng of cells in the presence of a pine needle matrix for ELISA and 100 ng/g of needles for the *P. scopiformis* DAOM 229536 assay. The pine needles did not interfere with the ELISA but have a significant effect on real-time PCR during the DNA

isolation and amplification steps. A considerable amount of cross reactivity with closely related species in the ELISA assays. The qPCR primers and probes had zero cross reactivity with all tested species, even very close relatives.

During the testing of tree samples, ELISA yielded a 7.7% positive rate for the 52 samples inoculated with *L. nitens* CBS 127941, the qPCR yielded a 58% positive rate. Tree samples inoculated with *L. nitens* CBS 127939 had a 7.0% positive rate by ELISA and a 38% positive rate by real-time PCR. The TaqMan qPCR assays developed for *P. scopiformis* DAOM 229536, *L. nitens* CBS 127941 and *L. nitens* CBS 127939 were more sensitive, rapid and easier to develop when testing white pine and white spruce needle samples. The assay cannot reliably quantify specific DNA without further study because of the interference with the conifer needle matrix during DNA isolation and amplification. It could be reliably used as a detection method in inoculated seedlings and trees when presence/absence data are required. The ELISA methods developed for *L. cf. piceae* CBS 127942, *L. nitens* CBS 127941 and *L. nitens* CBS 127939 would be more dependable for quantification but are labour intensive and expensive to develop.

Future developments of this work might be to develop PCR assays for other beneficial foliar endophytes inoculated into conifer seedlings to confirm the presence of the endophyte before the seedlings are planted in a tree stand. Methods to overcome the effect of the needle matrix by exploring alternative DNA isolation methods and assay optimizations are another future endeavour. The development of this qPCR rapid detection method for beneficial conifer

endophytes represents a significant improvement over existing methods and should be applicable to a broad range of future studies, with important implications for commercial forestry industry.

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