AN ASSESSMENT OF ENDOPHYTIC FUNGI IN NEEDLES OF THREE *PINUS* SPP. CULTIVATED IN SOUTH AFRICA

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Dissertation submitted in fulfilment of requirements for the degree of Magister Scientiae Agriculturae in the Faculty of Agriculture, Department of Plant Pathology of the University of the Orange Free State

By

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BLOEMFONTEIN

Trees are poems that the earth writes upon the sky.

We fell them down and turn them into paper that we may record our own emptiness.

▸ Kahlil Gibran ◂

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PREFACE

Pinus spp. are economically important to the forestry industry in South Africa. A wide variety of pathogenic fungi infecting pine trees poses a threat to the sustainable cultivation of this crop in South Africa. Many of these pathogens are known to have latent/endophytic phases of infection. The aim of the present study was to conduct a qualitative and quantitative survey of endophytic fungal populations of three commercially cultivated *Pinus* spp. in relation to certain environmental and host related factors.

This dissertation is a compilation of four manuscripts. The introductory chapter is a review of foliar endophytes and their interactions with host plants, with specific reference to the Gymnospermae. It discusses the nature of endophytic fungal relationships in various Gymnosperm hosts, and the factors that influence their colonisation frequencies within Gymnosperm foliage. The ecological role and specialisation of endophytes are also discussed.

Chapter 2 investigates the occurrence of endophytic fungi in needles of *P. patula, P. elliottii* and *P. radiata*. The results show a distinct colonisation pattern and species composition of the endophytic population. Reasons for specific patterns are discussed.

Results obtained from the investigation in chapter 2, lead to the initiation of the research in chapter 3. A more detailed study was undertaken to determine the biogeographical distribution patterns of endophytic fungi in two *P. radiata* trees of. One growing in an eight-year-old plantation, and the other, of the same age, but growing solitary in a nearby field. The effect of different microclimates and management practices, is reflected in both the frequencies and the

species composition of endophytic populations obtained from the needles. The role of true endophytes and latent pathogens in this context is also discussed.

The possible ecological role of certain endophytes in pine needles, and their substrate utilisation patterns, is discussed in chapter 4. Like pathogens, endophytes need certain enzymatic capabilities in order to enter plant tissue. The enzymatic capabilities of true endophytes was measured against that of known latent pathogens of *Pinus* spp. The conclusion was reached that endophytes, although possessing limited enzymatic capabilities, are nevertheless able to cause symptomless infections in their host plant. Possible reasons for this phenomenon are discussed.

Due to the fact that chapters represent manuscripts which are independent entities, some redundancy and a lack of continuity between chapters has been unavoidable. Where applicable, a citation is given indicating the names of the authors, journal of publication and current status of publication.

CHAPTER 1

FOLIAR ENDOPHYTES AND THEIR INTERACTIONS WITH HOST PLANTS, WITH SPECIFIC REFERENCE TO THE GYMNOSPERMAE*

ABSTRACT

This review discusses the nature of endophytic fungal relationships of the Gymnospermae and factors affecting their colonisation frequencies within Gymnosperm foliage. The role of fungal endophytes in insect herbivory, biological control, latent pathogenesis and other associations are addressed. Specific mention is made of host and fungal diversity, ecology of endophytic colonisation, and the physiology of endophytic associations. Aspects of quiescent infection, latent pathogenesis and absolute endophytism are also discussed.

Keywords: Endophyte, fungi, Gymnospermae, latent pathogen, *Pinus* spp., plant disease.

1. INTRODUCTION

Fungi live in a mutualistic, antagonistic or neutral symbiosis with a wide variety of both autotrophic and heterotrophic organisms. The properties of these relationships are diverse displaying varying degrees of association and nutritional interdependence (Petrini, 1986). Fungi living on the exterior of their hosts are called epiphytes, as opposed to those living within host tissue which are termed endophytes (De Bary, in Petrini, 1986). Endophytes, in contrast to epiphytes, are contained entirely within the host plant substrate, and may have either a parasitic or symbiotic

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association with the host (Sinclair and Cerkauskas, 1996). At the most basic level, "endophyte" simply refers to the location of the organism: "endo" means within, and "phyte" means plant, therefore describing all organisms that live inside a plant (Wilson, 1995a). The term has, however, evolved to indicate not only the location of the organism, but the actual type of association the fungi or bacteria have with their host. The nature of the interaction described by the term, endophyte, is that such organisms found inside the plants do not elicit symptoms of disease (so the infection is symptomless) (Wilson, 1995a).

Observations of asymptomatic fungal infections were made in various plant species as early as 1947 (Bose, 1947). Petrini (1986) postulated that all living plants probably host endophytes. The latter term describes all organisms that inhabit plant organs and can colonise internal plant tissues at some time in their life, without any immediate deleterious effect on their host (Petrini, 1991). This would also include endophytic organisms with an epiphytic phase and latent pathogens that may have a symptomless phase in their host. According to Wilson (1995a), "endophyte" describes the type of infection strategy. Kowalski and Kehr (1992) also introduced another term "phellophyte", for fungi typically colonising the dead outer bark tissue of tree stems. Endophytes from smaller woody organs such as leaves, petioles and twigs, were termed "xylotropic endophytes" by Chapela (1989). Carroll (1988) used the term endophyte to describe fungi that form inconspicuous or asymptomatic infections within the leaves and stems of healthy plants. Many endophytes are closely related to virulent pathogens, but have limited, if any, pathogenic effects on their host plants (Carroll, 1988). According to Dorworth and Callan (1996), the length of the latent endophytic stage is directly related to the extent of evolutionary advance or regression from the pathogenic to the mutualistic state. Endophytic pathogens (endophytic antagonistic symbionts), such as rust fungi have been studied extensively by plant pathologists (Petrini, 1986). In this review, the definition of endophyte, as circumscribed by Petrini (1991),

will be used.

According to Wilson (1995a) the most important question is not whether an organism is an endophyte or not, but why infection by endophytes does not trigger a defence response by the plant? Other important issues are: Why are they there? What are they doing? How do they affect the host plant? According to Wilson (1993), plants do not consist solely of plant tissues, and should be treated as evolving, integrated symbiotic units of plant and fungal cells, which can affect both ecological and physiological processes. Fungal endophytes thus have a very intimate and probably also a co-evolutionary relationship with their hosts, and thus, have the potential to influence the evolutionary trajectory of plant defences. Endophytes can for example protect host plants from insect herbivory (Clay, 1988; Clark et al., 1989) and other fungal pathogens (Carroll, 1988). They can therefore be used as bio-regulators to induce resistance against diseases; as biological control agents against certain pathogens (Bissegger and Sieber, 1994); and also in the biological control of undesirable weeds (Dorworth and Callan, 1996). Endophytes can also be used as bio-indicators, reacting to pollutants such as acid rain, ozone and industrial emissions (Helander et al., 1993b, 1996).

The occurrence of endophytes is not confined to the phanerogams, and seems to be quite common in pteridophytes (Dreyfuss and Petrini, 1984). A wide variety of coniferous tree species have yielded fungal endophytes (Carroll et al., 1977; Carroll and Carroll, 1978; Petrini and Müller, 1979; Petrini and Carroll, 1981; Petrini, 1986; Suske and Acker, 1987). The aim of this review is to investigate the endophytic fungal populations associated with Gymnospermae (needles, leaves, stems and roots of various species) so as to obtain a better understanding of the effects they may have on their host including aspects such as latent infection, pathogenesis, and possible beneficial associations.

2. DIVERSITY OF ENDOPHYTIC ASSOCIATIONS

In general, endophytes can be divided into two groups: firstly, those that are ubiquitous and can be isolated from a wide variety of host species in different ecological and geographical conditions, and secondly, species that show a fair degree of host specificity and follow the same patterns characteristic of obligate antagonistic symbionts (such as the Uredinales) (Petrini, 1986). Endophytes commonly isolated from a given host, and less frequently from other hosts are generally host specific. In contrast, endophytes that are rarely isolated from a given host species, appear to be less host specific and may be isolated from a wide variety of hosts (Petrini et al., 1982). Dreyfuss (In Bills, 1996) speculated that endophytic fungi represent one of the largest reservoirs of fungal species. According to Petrini (1996), "symptomless endophytes" can basically be assembled in two distinct ecological groups: the clavicipitaceous systemic grass endophytes, which live in a mutualistic symbiosis with their hosts; and the endophytes of trees and shrubs, including non-clavicipitaceous grass endophytes.

2.1. Diversity among host species (interspecific diversity)

Todd (1988) suggested that susceptibility to infection by endophytes is heritable, thus being a product of kin selection. According to Petrini and Carroll (1981), fungal endophytes displayed a degree of host specificity, at least at family level. This tendency may be more important than geographical location of the host plant as far as determining the overall distribution of endophytes. Host-specificity has shown to be directly correlated with the existence of a symbiotic association between a fungal endophyte and its host (Petrini and Carroll, 1981). Hata and Futai (1996) found the taxonomic position of host pine species, to have a strong effect on the mycobiota. In fact, taxonomy had a stronger effect on the distribution patterns of endophytic species in pines,

than factors such as sampling date, tree age and the location of the sampling tree (Hata and Futai, 1996).

Generally occurring endophytes such as *Epicoccum nigrum* Link and *Aureobasidium pullulans* (De Bary) Arnaud, are termed host-neutral endophytes (Boddy and Griffith, 1989) as opposed to an endophyte like *Rhabdocline parkeri* Sherwood-Pike, Stone and Carroll on Douglas fir [*Pseudotsuga menziessii* (Mirb.) Franco], which is absolutely host specific and has a close relationship with its only host (Sherwood-Pike et al., 1986). In two species of pine, namely *Pinus resinosa* Aiton and *P. banksiana* Lamb., commonly isolated endophytes showed a strong preference for their host (Legault et al., 1989). Planted stands of holly oak (*Quercus ilex* L.) lack characteristic species-specific endophytes that are found in natural stands (Fisher et al., 1994). Occasional isolation of host specific endophytes from other trees usually occurs only when these trees are in the vicinity of the main host (Kowalski and Kehr, 1996). The latter endophytes are able to colonise morphologically similar hosts growing at the same site. Petrini (1984) found that for ericaceous hosts, endophytes exhibited a moderate degree of host specificity.

Both qualitative and quantitative differences in infection frequencies of endophytes have been reported in specific host species. In extensively sampled conifer species, up to 110 (mean value = 60) fungal species could be isolated, with the majority (80-90 %) observed infrequently or only once (Carroll and Carroll, 1978). The total rate of infection in *P. sylvestris* L. was relatively high (80.1 %), whereas other *Pinus* species showed an infection rate of 20-100 % (Carroll et al., 1977; Petrini, 1986), and results of studies on five other pine species varied from 46.0 % to 92.3 % (Carroll and Carroll, 1978). Hata and Futai (1993) found a more extensive endophytic colonisation in *Pinus densiflora* Siebold and Zucc. than in *P. thunbergii* Parl. Kowalski (1993) isolated seven fungal species with an infection frequency of more than 5 % from symptomless needles of *Pinus sylvestris*, namely *Anthostomella formosa* Kirschst. (28.0 %),

Lophodermium seditiosum Minter, Staley et Millar (20.6 %), *Cyclaneusma minus* (Butin) Di Cosmo, Peredo & Minter (20.5 %), *Cenangium ferruginosum* Fr.: Fr. (15.7 %), *L. pinastri* (Schrad.ex Hook) Chev. (13.0 %), *Sclerophoma pythiophila* (Corda) Höhn. (6.4 %) and *A. pedemontana* Ferr. & Sacc. (5.5 %).

2.2. Diversity within host species (intraspecific diversity)

Factors inherent to the physiological condition of the host, eg. host genotype and age of foliage often play a significant role in the distribution of certain endophytes within the host itself (Todd, 1988). Old needles are more heavily colonised by endophytes than young ones (Bernstein and Carroll, 1977; Petrini and Carroll, 1981; Fisher et al., 1986; Sieber-Canavesi and Sieber, 1987; Stone, 1987; Hata and Futai, 1993; Kowalski, 1993). One exception to the tendency of increased frequency of infection with increased needle age, is *Anthostomella formosa*. This can be attributed to the low competitive ability of the fungus, and its inability to survive for long periods in needles, or the possibility that nutrients in older needles might become inadequate for its survival (Kowalski, 1993).

Infection frequencies of *Meria parkeri* Sherwood-Pike could be positively correlated with the growth speed of trees. *Trimmatostroma salicis* Corda was only found in the older needles of conifers, which could be attributed to the fact that wax layers on needles are weathered away during ageing (Millar, 1974). *Trimmatostroma salicis* grows and sporulates on the needle surface as an epiphyte, and due to the effect of the host ageing, it is consequently isolated frequently as an endophyte from older needles. In studies conducted with *Salicornia perennis* Mill., significant differences with regard to colonisation by different fungal species were found between old and new tissues (Petrini and Fisher, 1986). Fungi such as *Pleospora salicorniae* have been reported to colonise most parts of the host plant, but *Pleospora bjorlingii* was mostly confined to older plant

tissues. New tissues were colonised mainly by two species of *Stagonospora* and to a lesser extent by *Diplodina salicorniae* (Petrini and Fisher, 1986). Barklund and Kowalski (1996) found that the composition of endophytic species gradually changes, qualitatively and quantitatively, with the increasing age of internodes of Norway spruce (*Picea abies*). The most dominate species, *Tryblidiopsis pinastri* (Pers.: Fr.) P. Karsten, was most commonly isolated from young internodes, whereas three other common species, *Phialocephala scopiformis* Kowalski and Kehr, *Geniculosporium serpens* Chesters et Greenhalgh, and *Tapesia livido-fusca* (Fr.) Rehm were most frequently isolated from old internodes. These fungi, called phellophytes by Kowalski and Kehr (1992), were common in the older, thicker barked parts of the branch, which provide more protection for such fungi living near the surface. In contrast, *Tryblidiopsis pinastri*, which thrives on apical, thin barked parts of branches and could regularly be isolated from the inner bark, could therefore be described as a true endophyte. In comparison to other endophytes of Norway spruce, *T. pinastri* has a special relationship with this host revealing high levels of host specificity (Barklund and Kowalski, 1996).

As shown above, many endophytes are specific to the tissues and plant organs that they are able to colonise. Some fungi (e.g. *Acremonium* spp.and *Fusarium* spp.) are confined almost exclusively to roots, while others (e.g. *Pestalotia* spp.and *Colletotrichum* spp.) can be isolated only from aerial plant organs (Dreyfuss and Petrini, 1984). According to Fisher and Petrini (1990), different plant tissues and organs can be separated on the basis of their endophytic fungal populations. Fisher and Petrini (1987a) recorded 12 fungal species isolated from leaves and stems of *Suaeda fruticosa*. Of these fungi, *Colletotrichum phyllachoroides* (Ellis and Everh.) Arx. was confined to leaves, and two species of *Camarosporium* were isolated mainly from stems, with a higher incidence in whole stems, compared to isolations from the xylem. This demonstrated the ability of these fungi to penetrate deep into host tissue. Fisher and Petrini (1990) confirmed high

colonisation frequencies for bark and xylem of *Alnus* spp., but in general, the colonisation of experimental segments by more than two fungi is rare. Bissegger and Sieber (1994) found endophytes to be confined to the phellem in coppice shoots of *Castanea sativa* Mill., with no endophytic assemblages in the pith and xylem, and seldom in the bark tissues between the phellogen and cambium. Three to 16 endophytic thalli and one to six species were isolated per cm^2 of phellem tissue. The density of lenticels has had no influence on the frequency of colonisation, but the phellem adjacent to lenticels was more frequently colonised than the lenticels themselves. This could be attributed to the more intense surface sterilisation with the disinfectant having penetrated into the lenticels (Bissegger and Sieber, 1994). In studies done by Fisher *et al.* (1995) on *Dryas octopetala* L., a higher frequency of endophytic taxa was found in the leaves of the host than the twigs or roots. Endophytic fungi are also associated with non-ectomycorrhizal fine roots of forest trees and shrubs, and occur as dark, septate hyphae throughout the root tissue, except for the innermost phellogen (Ahlich and Sieber, 1996).

Sieber-Canavesi and Sieber (1987) observed no succession of endophytic species in needles of *Abies alba* Mill., in contrast to Carroll et al. (1977), who suggested succession in the endophytic petiole flora of *Sequoia*, and demonstrated that the needle petiole was more intensively colonised than the apex of the needle. Fungi associated with the petiole of *Sequoia* were similar to those commonly found in twigs, although they colonised only the cortex of twigs, and not the vascular bundles (Carroll et al., 1977). Infection frequencies of endophytic fungi were the highest at the needle base of some tree species (Bernstein and Carroll, 1977), but in pine needles it tended to be evenly distributed over the entire needle, with a slight increase at the middle section (Kowalski, 1993). Kowalski (1993) recorded distinct differences in differential species colonisation throughout the needle. This tendency varied between first and second year needles and can be attributed to different micro climatic conditions that prevailed in different needle

sections. The spread of fungi in needles was not only affected by the nutrient content and microclimate of the needles, but also the interaction between fungi, where some fungi such as *Sporormiella, Epicoccum, Cenangium, Lophodermium,* and *Coniothyrium* were able to limit the growth of other fungi (Kowalski, 1993). Substrate utilisation tests showed differences between the various fungi and their origin (Carroll and Petrini, 1983). In studies done on *Pinus densiflora* and *P. thunbergii,* Hata and Futai (1993) found a distinct distribution pattern of some of the dominant fungi, especially *Phialocephala,* at the proximal and more specific, the basal areas of *P. densiflora* needles. The higher colonisation frequency of endophytes in the basal part of the midrib of mountain birch [*Betula pubescens* var. *tortuosa* (Ledeb.) Nyman] leaves, could be explained by more favourable conditions created for spore germination, and higher levels of moisture and leachates (Helander et al., 1993a). Another possibility speculated by Helander et al. (1993a), concerns mycelia already present in the twigs, which might have grown into the leaf petiole, and eventually the leaf blade. In isolations of endophytic fungi from eastern larch [*Larix laricina* (Du Roi) K. Koch] leaves, no significant difference in the number of isolates could be detected between leaf segments from the petiole to the tip when all isolates were considered together (Dobranic et al., 1995). If one unidentified fungus was excluded from the analysis (by discounting its specific frequency), all the remaining isolates were isolated significantly more frequently from the petiole segment. Species composition in leaves of coastal redwood trees [*Sequoia sempervirens* (D. Don ex Lamb)], of progressing age in single branches, revealed a patchy pattern of colonisation, without showing any obvious sequence of succession (Espinosa-García and Langenheim, 1990). Endophytic populations in leaves and sprouts were very similar, however, showing distinct differences in species richness and distribution of certain fungal species such as *Pleuroplaconema* sp. and *Pestalotiopsis funerea* (Desm.) Stey. (Espinosa-García and Langenheim, 1990). Studies of the endophytic flora of sessile oak [*Quercus petraea* (Matt.) Lieb.] revealed a colonisation rate

of 97 % in leaves and 84 % in twigs. Leaves produced 78 different taxa, while the twig segments yielded 45. Of these taxa, 98 % belonged to the Ascomycetes or their anamorphs (Halmschlager et al., 1993). Fungal assemblages associated with American beech (*Fagus grandiflora* Ehrh.) and aspen (*Populus tremuloides* Michx.) were strongly dominated by Ascomycetes and Coelomycetes (Chapela, 1989).

2.3. Diversity among fungal species

The degree of host specificity among endophytes does not permit the use of endophytic distribution as a parameter of taxonomic affinity among various members of the same plant family. However, it could provide some useful taxonomic information if the parasites themselves were abundant and widespread (Carroll and Carroll 1978). In studies based on substrate utilisation tests and electrophoresis of soluble proteins and pectic enzymes, Sieber-Canavesi et al., (1991) found that three distinct species of *Leptostroma*, morphologically almost indistinguishable from each other, respectively colonised apparently healthy needles of *Picea abies* (L.) H. Karst., *Abies alba* and *A. balsamea* (L.) Mill. Many fungi from foliage of some Cupressaceae were isolated as anamorphs of known conifer-inhabiting Ascomycetes. The scarcity of Basidiomycetes in the endophytic flora could be more apparent than real, and might be due to the isolation and scoring methods used by researchers. Basidiomycetes tended to fruit infrequently in culture, and were therefore scored as "sterile" fungi in most instances (Petrini and Carroll, 1981).

Some endophyte species which have a large host range can be taxonomically differentiated into groups showing preference for specific hosts. *Discula umbrinella* (Berk. and Broome) Sutton, a common endophyte in leaves of Fagaceous trees in Europe and North America, showed distinct preferences for particular hosts (Toti et al., 1992). Isolates derived from beech trees could only adhere to, penetrate, and colonise beech leaves, and not the non-host leaves of oak and chestnut

trees in the way isolates from these hosts could (Toti et al., 1992). Hata (in Carroll, 1995) found various host-specific races or cryptic species of endophytes that existed between two *Pinus* spp., namely *P. thunbergii* and *P. densiflora*. Distinct patterns of endophytic colonisation were also detected in these needles. Considerable genetic diversity exists within natural populations of endophytic fungal species, as demonstrated by Wilson et al. (1994) for *Lophodermium pinastri* (Schrad.: Fr.) Chev. in *Pinus resinosa*. Different genotypes were also found among isolates of *L. pinastri* from the same tree. Frequently occurring endophytic taxa from *Alnus* spp. are morphologically identical, despite the different environmental conditions in which their host grow (Fisher and Petrini, 1990). McCutcheon and Carroll (1993) used Random Amplified Polymorphic DNA (RAPD's) to prove the genetic diversity between isolates of *Rabdocline parkeri* (anamorph of *Meria parkeri*) isolated from Douglas fir. The diversity was estimated to be at least three times greater in foliage of mature and juvenile trees in natural stands, compared to foliage from a managed stand or from an isolated tree. This could be attributed to the differences in tree age and accessibility of inoculum (McCutcheon and Carroll, 1993). A combination of cultural and biochemical data was used to determine taxonomic relationships of endophytic isolates of *Xylaria* species from *Euterpe oleracea* Mart. (Rodrigues et al., 1993). Because of taxonomic complications associated with *Xylaria* spp., criteria other than morphology had to be used to determine the taxonomic connections between different species. Isozyme analysis showed a high degree of variation within and among the putative species examined, which reflected the morphological variation found in pure cultures and confirmed the genetic diversity of the genus (Rodrigues et al., 1993).

3.ECOLOGY OF ENDOPHYTIC ASSOCIATIONS

3.1. The host plant: Gymnospermae

Coniferous foliage varies greatly in physical appearance, ranging from the needle-like foliage, which are typical of *Pinus*, *Abies* and *Picea*, to tiny, compressed leaves of *Cupressus*, *Thuja* and *Chamaecyparis*; and the rudimentary angiosperm-like leaves found on *Podocarpus*. While the aforementioned species usually retain their leaves for more than one year, *Larix* and *Metasequoia* are deciduous trees (Millar, 1974). Leaves are usually covered by a chemically complex, thick, waxy cuticle which can be covered with tubules. The cuticle may vary between and within species and consist of paraffin, ester and alcohol-soluble fractions and high carbon components (Schuck, in Millar, 1974). These waxes cover the whole leaf as well as the stomata, while forming an interlaced mat of tubules, influencing gaseous exchange of the plant (Jeffree et al., in Millar, 1974). These layers also prevent the direct entry of larger fungal spores (Millar, 1974), which in turn affects infection by endophytes and pathogens. The orientation, surface characteristics of the leaf surface and inoculum concentration reaching a particular host all effect infection, and ultimately fungal colonisation of the host (Fitt et al., 1989).

Changes in the ultrastructure of the leaf surface due to environmental factors such as air pollution, also have an effect on persistence of canopy moisture, which in turn will directly influence spore germination and growth (Helander et al., 1996). In studies done on larch trees, it was evident that the deciduous nature of these leaves resulted in a shorter period available for leaf colonisation, compared to the evergreen softwoods (conifers) (Dobranic et al., 1995). The major representative endophytic taxa were therefore also affected, and endophytes represented in larch leaves might be limited to those adapted for rapid leaf colonisation. The time needed to gain access to a particular host, and differences in leaf structure, should therefore be taken into

account when studying endophytic populations (Dobranic et al., 1995).

3.1.1. Physiology

Physiology of a host plant greatly influences its colonisation by endophytes. Essential oils in healthy leaves of coastal redwood [*Sequoia sempervirens* (D.Don ex Lamb.)] trees were an important factor controlling the activity of certain endophytes (Espinosa-García et al., 1993). A *Pleuroplaconema* sp., occurring in these redwood leaves, was stimulated by low essential oil doses, and inhibited by high doses. Essential oils were important inhibitors of *Pestalotiopsis funerea* (Desmaz.) Steyaert. However, other factors also involved in the inhibition process of fungi are still unknown (Espinosa-García et al., 1993).

3.1.2 Phenology

As discussed previously, needle age plays a significant role in the infection frequencies of endophytes (Todd, 1988). Knowledge of the seasonal development of a host, and its effect on needle age, can therefore be very illuminating in the understanding of endophytic colonisation patterns associated with that host. Whitehead et al. (1994) examined the seasonal development of the leaf area in young *Pinus radiata* D. Don plantations in New Zealand. The trees were 6 -7 years old, and elongation of age 0 needles (current year needle flush) began in the spring (October), and continued through summer, becoming fully elongated during autumn (early May), approximately 200 days from the onset of elongation. A smaller growth flush started in summer (January), and needles elongated until the end of the growing season. No significant difference in needle density could be detected with change in canopy height or seasonal variation. Needle density would affect the microclimate and inoculum distribution among needles. Needles of the age 1 group (previous years' needle flush) started to decline during midsummer (end of January),

and coincided with the time of maximum elongation of age 0 needles. Needles formed during the spring growth flush contributed the majority of new leaf area during the year, with only a small proportion added by the autumn flush, which occurred predominantly on branches at the top of the canopy. The age of these needles and the climatic factors during needle development, would therefore affect the succession of endophytes in needles. Researchers also believe that needle longevity would increase with stand age (Whitehead et al., 1994), and therefore provide a suitable niche for true endophytes.

3.2. The Endophyte

3.2.1. Authenticity of the endophytic character

Petrini (1984) examined the dependability of the endophytic character of some of the coprophilous fungi isolated as "endophytes" from ericaceous hosts. He determined that the surface sterilisation techniques used were extensive enough to ensure the genuine endophytic character of even these coprophilous fungi. According to Carroll et al. (1977), the sporadic isolation of *Aureobasidium pullulans* (de Bary) G. Arnaud from conifer needles, could be contributed to contamination from epiphytic fungi. Pugh and Buckley (1971), however, frequently isolated endophytic *A. pullulans* from surface-sterilised living twigs, buds, leaves and seeds of sycamore (*Acer pseudo-platanus* L.), and from twigs of horse-chestnut and lime. Most common endophytes are seldom collected in the field, because they rarely sporulate on their hosts or form inconspicuous fruiting bodies (Petrini, 1986). Frequently occurring endophytes from a given host were absent among epiphytes, and likewise, epiphytes were uncommon among endophytes (Fisher and Petrini, 1987b). The fact that endophytes were absent among epiphytes, could be attributed to the methods of isolation, which tend to favour fast growing saprophytes, or in this case epiphytes. Epiphytes, on the other hand, were excluded from endophytic isolations

due to extensive surface sterilisation.

According to Kowalski and Kehr (1996), endophytes may have the same importance for trunk and branch tissues that mycorrhizae have for the roots. Primary characteristics of mutualistic symbiosis, include the lack of cell or tissue destruction, recycling of nutrients or chemicals between the fungus and the host, enhanced longevity and photosynthetic capacity of infected tissues, enhanced survival of the fungus, and a tendency of greater host specificity than is evident in biotrophic infections (Lewis, 1973). Endophytes are contained within the plant, and may be either parasitic or symbiotic. True endophytic colonisation or infection is asymptomatic and can be described as a mutualistic symbiosis, which includes a lack of destruction of most cell tissues, nutrient or chemical cycling between host and fungus, enhanced longevity and photosynthetic capacity of infected tissues, and enhanced survival of the fungus. Endophytic infections can therefore not be considered as causing disease, because plant disease is an interaction between the host, parasite, vector and the environment over time, which results in the production of disease symptoms and/or signs (Sinclair and Cerkauskas, 1996). The distinction between endophyte and pathogen is not always clear, as some diseases are characterised by a long retardation in the development of progressive disease, due to the growth of the potential pathogen being arrested (Swinburne, 1983). This gives rise to latent infection, where the distinction between *bona fide* endophytes and latent pathogens become more confused.

3.2.2. Sporulation, dispersal and infection

Endophytes can be transmitted from one generation to the next through host seed or vegetative propagules (Carroll, 1988). In this instance it is referred to as a systemic infection or as described by Wilson (1996), vertical transmission. Horizontal transmission occurs when infection of leaves or needles takes place by means of spores, and these infection levels are closely correlated with the

seasonal distribution of rainfall (Wilson, 1996). Inoculum dispersal of infectious fungi can be divided into three stages: removal from the colonised substrate, transport through air, and deposition on a new host. Rain and/or wind may be involved in all three stages and the two modes are not mutually exclusive (Fitt et al., 1989). Spores from fungi that produce their spores in mucilage, are detached from the host by raindrops and dispersed in splash droplets (Fitt et al., 1989). This includes conidia of some endophytic fungi produced in gloeoid masses, which have been encountered in through fall samples collected in coniferous stands (Carroll and Carroll, 1978). When canopies become saturated by rain, fog, dew, or mist, large drops may form on the leaves and under canopies, drip-splash may be as important as direct rain-splash (Fitt et al., 1989). Survival stages of endophytes are often present on litter trapped between branches within the tree canopy, from where the spores are subsequently dispersed by wind or rain (Carroll et al., 1977). Rain consisting of large drops is the most effective means of dispersing spores. Drops from the canopy foliage can also be effective because they are often large, but their falling speeds are less than their terminal velocity (Fitt et al., 1989). The mucilage surrounding splash-borne spores protects them from desiccation and loss of viability during dry weather. This may confine the dispersal of certain fungal spores to periods of rainfall when conditions are also favourable for infection because of the availability of free water on the host surface.

Wind is also an important factor in the dispersal of certain fungal spores, especially hyphomycetes. Some fungal spores are actively removed from the host by turbulent winds, and since most endophytes sporulate on litter trapped between branches in the tree canopy, their dispersal should be affected by wind or rain within the canopy (Carroll et al., 1977). Although average wind speeds in the lower part of closed canopies are typically only a fraction of the speed above the canopy, gusts of wind with speeds several times higher than the local mean may occur frequently enough inside plant canopies (Aylor, 1990). In general, spores produced in the lower

part of the canopy are exposed to slower wind speeds and less turbulence. This lower amount of turbulence may prevent the escape of large numbers of spores from a closed canopy (Aylor, 1990). This will affect the distribution of fungal endophytes within the canopies of host plants.

Hata et al. (1998) also provided other ways in which endophytic infections may take place. Mycelia of the endophytes *Phialocephala* and *Cenangium ferruginosum* may infect current-year needles of *Pinus thunbergii* and *P. densiflora* via current-year twigs in early summer and *Leptostroma* infect the needles with spores via the needle sheath (Hata et al., 1998).

3.2.3. Colonisation

Todd (1988) found that there was a direct correlation between site and the infection frequencies of endophytes. This could be attributed to; (i) a microclimate more conducive to fungal colonisation where the foliage was more dense; (ii) the relative position of the needles in the canopy; or (iii) other unknown factors. Theoretically, needle infections can originate from systemic infections in twigs and petioles, through penetration of the cuticle or stomata by mycelium of fungi from epiphytic origin, multiple infections by airborne and/or waterborne spores, or through inoculation by various sucking insects (Bernstein and Carroll, 1977). Bernstein and Carroll (1977) suggested that 1-year-old needles became infected by waterborne spores dispersed by rain. Infection thus increases with needle age and the availability of rainfall during the fruiting stages of endophytic fungal species, which is in contrast to needle pathogens, where most infections are confined to young needles (Carroll, 1995). Other possibilities are that of a systemic infection as in *Guignardia philoprina* in *Taxus* needles (Carroll et al., 1977) and seed transmission. The life cycle of seed-borne endophytes is inexorably tied to their grass hosts (Wilson, 1993).

Rhabdocline parkeri (teliomorph of *M. parkeri*) an endophyte of Douglas fir, infects healthy foliage by direct penetration of the host epidermal cell walls, accomplished by very fine penetration

hyphae (Stone, 1988). According to Sherwood-Pike et al. (1986), the fungus can persist in living host needles for up to 4 years. These intracellular hyphae occupy the entire lumen of a single epidermal or hypodermal cell (Sherwood-Pike et al., 1986), which eventually leads to the death of the colonised cell (Stone, 1988). Although the hyphae do not elongate, they appear to be metabolically active. At the onset of needle senescence, haustoria are produced from the intercellular hyphae (Stone, 1988), so that rapid colonisation and sporulation can occur immediately after abscission (Sherwood-Pike et al., 1986). The micro conidial anamorph is the first state to be produced by *R. parkeri*, followed by the *Meria* state, which is rapidly produced in the same conidioma. The function of the microconidia is still unknown, but the macroconidia serve to reinfect the host plant (Sherwood-Pike et al., 1986).

Cabral et al. (1993) found characteristic mechanisms of penetration and colonisation of individual fungal species in the tissue of *Juncus* spp. Infections of *Stagnospora innumerosa*, a *Drechslera* sp. and an unidentified endophyte of *J. bufonius*, were limited to a single host epidermal cell. *Phaeosphaeria junicola* (Rehm) L. Holm. infected the substomatal cavity of *Juncus* leaves, followed by limited intercellular colonisation of the mesophyll. Infections by *Cladosporium cladosporioides* (Fresen.) G.A. De Vries and *Alternaria alternata* (Fr.: Fr) Kiessl. are localised to the substomatal chambers, and only *A. alternata* will colonise the mesophyll tissue intercellularly. The colonisation patterns of these two endophytes are typical of opportunistic saprophytes (Cabral et al., 1993). Stone et al. (1994) suggested that active host defences, triggered by initial invasion of endophytes, are probably responsible for the restriction of endophytic colonisation, but little evidence for such a response exists.

Ascospores of fungi in the Xylariaceae (mostly endophytes) are irreversibly activated for infection, prior to germination, within minutes of contacting a potential host. These spores are able to recognise different plant species due to their ability to distinguish between structurally

similar monolignols (Chapela et al., 1991). This suggests the existence of a host-specific "signature" present on different plants, and specific receptors for these molecules, within the fungal spores (Chapela et al., 1991).

Ascomata of two Norway spruce endophytes, *Tryblidiopsis pinastri* and *Lophodermium piceae* only develop several years after initial colonisation on dead branches and needles, respectively (Barklund and Kowalski, 1996). In contrast, an *Ophiognomonia* sp. which is an endophyte of *Quercus emoryi* Torr., naturally occurs at very high levels, but is only present in the leaves for the last 3-4 months before leaf abscission (Wilson, 1996). According to Carroll (in Wilson, 1993), the co-occurrence of senescence and endophyte growth, could lead to competition between the plant and endophyte for the mobilised nutrients destined for recycling inside the host plant. Persistence of endophytic fungal mycelia originating from latent infections in decomposing tissues, will depend on their ability to utilise changing energy and nutrient sources, tolerate changing microclimatic conditions, and to defend their territory against invasions by other primary or secondary colonisers (Boddy and Griffith, 1989). Leaf senescence is the process which precedes tissue death, and during which the photosynthetic activity in leaves stops and leaf constituents are broken down and recycled within the host plant (Wilson, 1993). This process is followed by abscission, colonisation and decomposition by saprophytic fungi. Endophytic fungi present in these healthy leaves will be the first to capitalise on the senescing and abscised leaves, and therefore the first species on the decomposing succession ladder (Wilson, 1993). Leaf senescence may trigger the growth and colonisation of endophytes, but endophyte growth may also trigger the onset of senescence. Heavy fungal infections of *Schizothyrium* sp. in needles of Douglas fir, resulted in premature senescence and abscission of needles (Sherwood and Carroll, 1974). In contrast, the infection frequencies of needle endophytes such as *R. parkeri*, was found to increase continuously with needle age, until colonisation resumes at the onset of needle senescence

(Stone, 1987).

The endophytic phase of branch pruning fungi can give them some advantage in colonising dying branches (Kowalski and Kehr, 1996). Almost all living branches of eleven deciduous and coniferous European tree species investigated by Kowalski and Kehr (1992) were colonised by some species of highly specific fungal endophytes. Most of the common branch pruning fungi found in general, were present in living branches, and therefore have an advantage in colonising the dying tissue (Kowalski and Kehr, 1992). Primary colonisers of dead or attached twigs derive considerable benefit from their endophytic habit, which allows them to respond rapidly to twig death and establish themselves in the resource before the arrival of secondary colonisers (Boddy and Griffith, 1989). Some branch pruning fungi, however, are not adapted to endophytic life and are frequently found in wood of dead, debarked branches, and are not isolated from living branches. Other fungi are totally adapted to an endophytic lifestyle, but are not able to colonise branches extensively after they die. Thus, it may be speculated that these fungi require more constant moisture conditions in the form of larger branch diameters and stumps in order to become established in the succession of decay fungi (Kowalski and Kehr, 1996).

3.2.3.1. ENVIRONMENTAL FACTORS INFLUENCING COLONISATION

Changes in the environment can influence plants by altering the interactions between microbial symbionts (such as endophytes), plant pathogens and herbivores (Helander et al., 1996).

Air pollution affects trees directly by damaging needles and leaves and causing a decrease in the assimilative capacity of the canopy (Helander et al., 1996). Indirect effects occur via the soil, due to acid rain that changes the nutrient content of the soil and causes the accumulation of hazardous ions. Microfungi living inside aerial plant parts can thus be affected and changes in the species composition of these microfungi may have various consequences for other role players in

the ecosystem, such as the host plant, plant pathogens and herbivorous insects (Helander et al., 1996).

Endophytic fungi live most of their life cycle in an environment protected against sudden weather changes and various environmental factors, including air pollution. Air pollutants, however, modify the microhabitat of the leaf surface, and can affect spore germination and hyphal penetration. In the light of this, several researchers have suggested that endophytes can serve as bio-indicators of air pollution. Sieber (1989) suggested that air pollutants are possible causes of changes in endophytic populations of *Picea abies* (L.) Karst. and *Abies alba* Mill. in Switzerland. The design of the experiment did, however, not allow the effects of air pollutants to be quantified. Helander et al. (1994) studied the effects of simulated acid rain on the occurrence of endophytic fungi in needles of Scots pine (*Pinus sylvestris*) from the sub-arctic region where environmental pollution is low. The frequency of endophytic colonisation was reduced on pines treated with spring water acidified with either sulphuric acid alone, or in combination with nitric acid. Nitric acid alone had no effect on endophytic colonisation (Helander et al., 1994). Simulated acid rain was also shown to affect the frequency of endophytic colonisation in leaves of mountain birch, with a 25 % decrease after an acid rain treatment at pH3. Species composition, however, was not affected (Helander et al., 1993a). Ozone (O_3) also has an effect on endophytic colonisation. The most common fungal endophyte isolated from Sitka spruce [*Picea sitchensis* (Bong.) Carrière] needles, *Rhizosphaera kalkhoffi* Bubák, was found to be increased by O_3 exposure (Magan et al., 1995). The same fungus showed a trend to increase under higher sulphur dioxide (SO_2) concentrations, although this was not statistically significant. Laboratory studies done by Smith (In Magan et al., 1995), suggested that *R. kalkhoffi* is tolerant of elevated SO_2 concentrations and the low availability of water, enabling it to compete more effectively in comparison with other needle phyllosphere or endophytic fungi. The general occurrence of *Lophodermium* species on

Scots pine needles was related to the distance from factory complexes producing copper, nickel, sulphuric acid and fertilisers, and to the chemical composition of living needles (Helander et al., 1996). The adverse effect of air pollution was the clearest in the most abundant species, *L. pinastri* (Schrad.: Fr.) Chev. The decrease in *Lophodermium* species can probably be contributed to the toxicity of industrial emissions, such as heavy metals, but impoverished vegetation and its associated changes in the microclimate, may have played an additional indirect role in endophytic fungal colonisation. Helander et al. (1996) found the number of endophytic fungi in pine needles to be consistently lowest in high intensity acid rain treatments. In general, however, endophytic fungi are protected from the effects of environmental changes such as air pollution, when compared with epiphytic microorganisms, but if endophytic communities are affected by a long term exposure to pollutants, the change may be more permanent, with implications to resistance and basic tree health for foresters (Helander et al., 1996).

3.2.3.2. SPECIES COMPOSITION AND CANOPY CHARACTERISTICS

Differences in composition of the endophytic flora in branches of forest trees can be caused by several factors. The diversity of the plant community may greatly influence the extent of colonisation by endophytes, and is illustrated by the occurrence of host-specific fungi on non-hosts, growing in mixed stands together with the main host (Kowalski and Kehr, 1996). Species composition in the endophyte population in *Abies alba* is dependant on the management type of the forest (Sieber-Canavesi and Sieber, 1987). Clear cuttings and plantations eliminate the transmission of endophytic fungi and clear cutting modifies the plant community as well as the microclimate. Where trees arise spontaneously, needle endophytes are found more frequently than in cases where they have been planted (Sieber-Canavesi and Sieber, 1987). Studies conducted on endophytic fungi present in foliage of different Cupressaceae in Oregon revealed differences

in the infection rates of endophytes (Petrini and Carroll, 1981). These studies included samples from *Calocedrus decurrens* (Torr.) Florin, *Juniperus occidentalis* Hook., *Thuja plicata*]. Donn ex D. Don and *Chamaecyparis lawsoniana* (A. Murr.) Parl. Samples taken from pure stands of any particular host showed higher infection rates than those took from mixed stands with an open canopy. This was confirmed by Legault et al. (1989) in subsequent studies done on *Pinus banksiana* and *P. resinosa,* which showed a higher colonisation rate in a closed canopy. Helander et al. (1996) found different results in Scots pine needles, where pine needles of trees having few other pines in their vicinity beared none or only few endophytic fungi.

Two types of endophyte dynamics were reported by Widler and Müller (1984): (i) fungi showing an increased frequency of occurrence with leaf age, and (ii) fungi showing a decreased frequency of occurrence with leaf age. Hata et al. (1998) isolated endophytes from needles of *P. densiflora* and *P. thunbergii.* The two most frequently isolated fungi were the *Leptostroma* anamorph of *Lophodermium pinastri* and *Phialocephala* sp. *Leptostroma* showed increased frequencies with needle aging and *Phialocephala* decreased frequencies. Possible explanations for the increase in *Leptostroma* with needle aging are (i) an increased chance of infection with the time after needle flush, (ii) improved habitat condition with the changing needle physiology with needle aging, and (iii) an increase in microscopic wounds or changes in the physical conditions of needles which may facilitate fungal infection. Hata et al. (1998) rated (i) and (ii) the most probable explantions. Probable factors contributing to a decrease in the detection frequency of *Phialocephala* with needle aging, are (i) earlier fall of needles colonised by *Phialocephala,* (ii) aggravation of habitat condition for the endophytes with the changing physiology due to needle aging (such as an increase in antifungal substances), and (iii) competition with other fungi, such as *Leptostroma.* Hata et al. (1998) found (iii) to be the most probable, since *Leptostroma* and *Phialocephala* showed antagonistic interaction in culture. In any particular tree, general infection

rates of endophytes increase with increasing age of foliage and decreasing distance from the tree trunk (Petrini and Carroll, 1981). The height of the needles in the tree canopy showed little correlation with the frequency of infections and latent fungal infections were present in all needles examines older than 3 years (Bernstein and Carroll, 1977). *Pinus* spp. showed higher colonisation rates with increasing foliage age, but it was not influenced by twig orientation (Legault et al., 1989). Sherwood and Carroll (1974) found parasitised needles to be shed from trees prematurely, because results showed a drop in the infection frequencies in needles from old-growth (7 - 8 yr) of Douglas fir. Four-to-five-yr old needles were most severely infected. Overall intensity of infection did not, however, increase with age or canopy level (Sherwood and Carroll, 1974). More endophytes could be isolated from the lower branches (up to 1 m) of mountain birch, than from branches at 2 m height, which is possibly due to the inoculum pressure and more favourable microclimate in the lower parts of the canopy (Helander et al., 1993a). More endophytes were isolated from the bottom of the crown in *A. balsamea*, than from the top, but no correlation could be found between the frequency of infections by endophytes and the geographic directional orientation of needles (Johnson and Whitney, 1989). This could be due to the availability of leachates and water within the crown (McBride and Hayes, 1977). The distribution correlates with the movement of propagules from the top to the bottom of the tree, and the fact that most endophytes are dispersed through water-borne spores (Johnson and Whitney, 1989).

3.2.3.3. GEOGRAPHIC AND CLIMATIC FACTORS

Geographical and local site factors apparently influence the composition and frequency of host-specific fungal species (Kowalski and Kehr, 1996). Changes in endophytic infection rates may be the result of various environmental changes rather than just direct or indirect effects of air pollution

and other factors (Helander et al., 1996). According to Carroll (1995), general exposure and geographic continuity are a significant factor when overall endophyte assemblages in a given host are compared over several dispersed sites. Carroll and Carroll (1978) suggested that low infection rates seen at high elevations and dry sites could result from the delayed onset of endophytic infections and not lower incidences of internal needle fungi per se.

Endophytic infections are influenced by precipitation and elevation. Precipitation in the form of rainfall may be a factor in endophyte dispersal, where moist sites show higher incidences of endophytic infections than dry sites. Petrini et al. (1982) proved that the infection rates of endophytes for a specific host species correlate positively with the relative canopy density and the moisture available at the collection site. Carroll and Carroll (1978) found that a lack of rain and relatively open conifer stands may limit the spread of endophytes. Sites which receive less rain and more snow (usually at higher elevations) will also result in a negative correlation between endophyte incidence and elevation (Carroll and Carroll, 1978). Carroll and Carroll (1978) also found the infection frequencies of endophytes to decrease with increasing elevation on western slopes and to increase with increasing elevation on eastern slopes, and explained this by differences in the amount as well as the form of precipitation. Bernstein and Carroll (1977) couldn't find any correlation between the internal canopy infections of endophytes of Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco] foliage, and the elevation and exposure of individual trees sampled.

Between site differences in the frequency of colonisation of *Castanea sativa* Mill. by *Amphiporthe castanea* (Tul.) Barr and a *Phomopsis* sp. could probably be attributed to differences in climatic factors and abundance of inoculum (Bissegger and Sieber, 1994). Samples of leaves, twigs and roots of *Dryas octopetala* taken in the subalpine region, are richer in endophytic species than samples collected in the alpine or Arctic regions (Fisher et al., 1995).

According to Widler and Müller (1984) endophytes show seasonal patterns in four

categories with regard to their distribution in leaves: (i) fungi that appear once a year for a short period, (ii) fungi with a higher frequency in winter than in summer, (iii) fungi with a higher frequency of occurrence in summer than in winter, and (iv) fungi which do not show any apparent seasonal change. Fungi that show high colonisation frequencies can usually be classified into the fourth category (Widler and Müller, 1984). In general, infection frequencies of endophytes seem to be higher in winter than in spring (Carroll et al., 1977). Hata and Futai (1993) found that the colonisation rate of endophytes increases with advance of the season, and even differs from year to year. This tendency possibly reflects changes in needle physiology and changes in biotic and abiotic environmental factors such as other micro fungi and climatic elements. Kowalski (1993) found winter to be an inhibiting factor for the infection of endophytes, and therefore fewer endophytes were isolated during spring and summer than in autumn. This could be explained by a lower chance for infection during winter. In contrast, Sieber-Canavesi and Sieber (1987) found a higher infection frequency in *Abies alba* needles during winter from especially endophytes of the Xylariaceae. This was attributed to the lower physiological activity of trees, resulting in a slower reaction of trees to fungal infection, and possibly enhanced penetration due to frost damage to the needle cuticle (Sieber-Canavesi and Sieber, 1987).

3.2.4. Substrate utilisation

Endophytes may develop distinct substrate utilisation patterns. For instance fungi from needle bearing conifers show specialisation in their utilisation capabilities. Fungi occurring only in the petioles have a broad range of substrate utilisation capabilities, but those occurring in the needle blades have more restricted abilities (Carroll and Petrini, 1983). Even isolates from the same fungal species may differ in their substrate utilisation. Differences in utilisation also ensure that several endophytes can coexist within a single needle, without competing with each other. This

is called "biochemical partitioning of resources" (Carroll and Petrini, 1983).

Pectin can be utilised by almost all fungi, lignin to a limited extent by needle fungi, but not by petiole fungi. Only petiole fungi are able to break down cellulose, hemicellulose, lipids, pectin, xylan, mannan and galactan, which suggest that they are active decomposers, whereas needle fungi not able to utilise some of these complex substrates are dependent on their host for their simple carbon sources (Carroll et al., 1977; Carroll and Petrini, 1983). Carroll and Petrini (1983) suggested that endophytic fungi with restricted substrate utilisation capabilities (like needle blade endophytes), are the most likely to have possible symbiotic relationships with their host plants. Fungi with broader substrate utilisation patterns (like petiole endophytes), are more likely to be latent pathogens. Endophytes capable of utilising only the simple carbon sources in living plant cells, will decline rapidly with the depletion of the food source following the death of the host tissues (Boddy and Griffith, 1989). Endophytes which commonly occur in healthy twig bark but are absent in dead wood, have a limited capacity to utilise complex substrates, in particular lignocellulose. These endophytes are dependant upon their host for simple carbon compounds (Boddy and Griffith, 1989).

Substrate utilisation and growth experiments have no taxonomic relevance for the distinction of some endophytic species, as was shown for conifer inhabiting *Phyllosticta* species. However, a comparison of the electrophoretic banding patterns of different enzymes such as pectinase, polygalacturonase, and amylase, nonetheless, allowed a clear differentiation between five *Phyllosticta* spp., namely *P. multicorniculata* Bisset et Palm, *P. cryptomeriae* Kawamura, *P. abietis* Bisset et Palm, *P. pseudostugae* L.E. Petrini and *Macrophoma piceae* L.E. Petrini (Petrini *et al.*, 1991).

4. HOST-ENDOPHYTE INTERACTIONS

4.1. Mutualistic associations

Although the ecological statuses of many endophytes remain undefined, possible benefits of endophytes to coniferous hosts include antagonism towards pathogenic needle parasites and surface saprophytes, delay in needle senescence, and a decrease in needle palatability for grazing insects (Carroll and Carroll, 1978).

4.1.1. Resistance to diseases

Phytoalexin production by the host plant in reaction to infection by an endophyte can actually render the host resistant to attack by pathogens (Wilson, 1993). The absence of endophytes in greenhouse raised plants may therefore explain their acute susceptibility to insect and fungal pests and diseases, since these plants are protected against natural airborne inoculum of endophytes (Wilson, 1993).

Mutual exclusion of endophytes within leaves where infection by one species may inhibit infection by another is also documented. For example, leaves sprayed with *Asteromella* sp. or *Plectophomella* sp., which are recognised endophytic fungi, were able to exclude other endophytic fungal infections (Wilson, 1996). *Cryptosporiopsis abietina* is a stem endophyte of *Picea sitchensis*, and shows antagonistic activity against *Heterobasidion annosum* (Fr.: Fr.) Bref. The fungus also behaves as an aggressive seedling pathogen on *Picea abies* and can be associated with declining mycorrhiza (Holdenrieder and Sieber, 1992). Bissegger and Sieber (1994) also isolated from European chestnut a fungus with antifungal properties, related to *Cryptosporiopsis*, namely *Pezicula cinnamomea* (DC.) Sacc. *Pezicula cinnamomea* inhibited other pathogens, including *Cryphonectria parasitica* (Murrill) Barr in dual cultures, possibly rendering it as an effective natural biocontrol

agent (Bissegger and Sieber, 1994). Due to the fungitoxic effects of *Balansia cyperi* Edgerton, an endophyte of *Cyperus rotundus* L., this fungus is able to exclude pathogens such as *Rhizoctonia solani* Kühn, from the leaves of its host (Stovall and Clay, 1991). *In vitro* bioassays with mycelium and culture filtrates of *B. cyperi* showed inhibition of test fungi which included *Fusarium oxysporum* Schlechtend.: Fr. and *R. solani*. Solvent extracts made of leaves from *B. cyperi* infected plants, also inhibited the growth of fungi including *F. oxysporum*, *Rhizoctonia oryzae* Ryker and Gooch and *R. solani*. These results show the possibility of *B. cyperi* to prevent infection of *C. rotundus* by other pathogenic fungi (Stovall and Clay, 1991).

Secondary metabolites produced by fungal endophytes in tomato roots are highly toxic to *Meloidogyne incognita*, especially strains of *Fusarium oxysporum* (Hallmann and Sikora, 1996). These toxins were produced by a nonpathogenic strain of *F. oxysporum* and were highly effective towards sedentary parasites and less effective towards migratory endoparasites, while nonparasitic nematodes were not influenced at all. Metabolites of this fungus also reduced the growth of pathogens such as *Phytophthora cactorum* (Lebert and Cohn) J. Schröt., *Pythium ultimum* Trow and *Rhizoctonia solani* in *in vitro* studies (Hallmann and Sikora, 1996).

Biological control of certain diseases, such as chestnut blight caused by *Cryphonectria parasitica* on *Castanea sativa*, can be obtained by spreading hypovirulence by means of endophytic thalli from hypovirulent strains of *Cryphonectria parasitica* (Bissegger and Sieber, 1994).

4.1.2. Protection from insect herbivory

Endophytic fungi can affect the interaction between their hosts and insect herbivores. Where a mutualistic association exits between fungi and insects, it will result in increased herbivory of host plants, and a mutualistic association between fungi and plants, in reduced herbivory of the host plant, as is found in grass endophytes (Clay, 1987). When the endophyte-plant symbiosis is

strongly mutualistic and the host benefits through increased defence against herbivores, the host may rely largely or wholly on the endophytes for their resistance (Wilson, 1993). Endophyte-infections therefore provide a selective advantage to grazed plants. There are four different mechanisms by which these fungi can influence herbivory of grass hosts; (i) by changing the consistency of host tissues, (ii) inducing resistance, (iii) depletion of nutrients, and (iv) the production of certain toxins (Clay, 1987). Systemically infected grasses display an increased level of resistance to a wide variety of insect as well as mammalian herbivores as a result of alkaloid production by fungi (Clay, 1987). The most clear cut mutualistic association is that of *Balansia* spp., which produce substances capable of reducing the palatability of the grasses to various herbivores (Clay, 1988).

There are conifer endophytes that have evolved an ecological strategy that involves the production of compounds that limit the herbivory of conifer needles (Clark et al., 1989). This suggests a mutualistic relationship between the fungus and its host. Infection levels of specific endophytic fungi (with beneficial associations) can be effectively manipulated using polyethylene or PVC bags to exclude other organisms. Inoculation of the leaves with specific endophytic fungi can be done by spraying spore suspensions onto the protected leaves (Wilson, 1996). Certain endophyte species inhabiting conifer needles produce compounds that could be linked to the mortality or decreased growth of spruce budworm larvae (Clark et al., 1989). Some species are in the genus *Leptostroma*, but the most toxic strains are not yet identified and could represent new genera. These coniferous endophytes produce compounds that either effect spruce budworm, mortality, and retard larval development (Clark et al., 1989). This can have important ecological consequences, and could result in the disruption of mating because affected budworms reach pupation much later than unaffected worms. Furthermore, larvae will be exposed to adverse environmental and predatory factors for longer periods, and thus suffer a higher mortality. The

occurrence of "escaper trees" in budworm-damaged forests could be attributed to the presence of these endophytes (Clark et al., 1989). Calhoun et al. (1992) refined and identified four toxic metabolites produced by endophytes of balsam fir which are effective against spruce budworm. Three compounds produced by *Phyllosticta* sp., are called (i) heptelidic acid, (ii) heptelidic acid chlorohydrin, and (iii) hydroheptelidic acid. A fourth compound, (+)rugulosin, an anthraquinone, is produced by *Hormonema dematioides*, and exhibits a wide spectrum of biological activity (Calhoun et al., 1992).

The most important endophyte of Douglas fir, *Meria parkeri* Sherwood-Pike, produces compounds toxic to insects (Todd, 1988). Diamandis (1981, in Gange, 1996) found the larvae of the pine processionary moth (*Thaumetopoea pityocampa*) to avoid endophyte-infected needles of *Pinus brutia*. Insect death can also be contributed to starvation in the case *Quercus garryana*, where the endophytic fungus kills the galls of a cynipid wasp, and deprives the insects of food (Wilson, 1995b). Endophytic fungi in galls caused by the pine needle gall midge [*Thecodiplosis japonensis* Uchida and Inouye (Diptera: Cecidomyiidae)], show distinct differences from endophytes isolated from healthy needles (Hata and Futai, 1995). A *Phialocephala* sp. was the most frequent endophyte occurring in the base of needles and galls from *Pinus densiflora* and an F_2 hybrid pine (a cross between *P. thunbergii* and *P. densiflora*). However, species richness increased in the gall infested needles. Hata and Futai (1995) suggested that fungi occurring in gall infested and healthy needles could represent different ecological groups of endophytes. Endophytes from healthy and gall infested needles can be divided into two groups: position-specific fungi such as *Phialocephala* sp. and *Leptostroma* spp., which showed a distinct pattern in their needle distribution; and gall-specific fungi such as *Phomopsis* sp., *Pestalotiopsis* sp., and to a lesser degree *Alternaria alternata*, which preferred galls on infected needles (Hata and Futai, 1995). No mutualistic associations between the gall endophytes and the pine needle gall midge could be

detected, and no evidence was found of transmission of endophytic fungi by the gall midges (Hata and Futai, 1995).

4.1.3. Growth promotion

Some endophytes promote growth of their host plants. *Leptodontium orchidicola* Sigler and Currah, a dematiacious hyphomycete isolated from roots of subalpine plants, caused a significant increase in host root length of *Salix glauca* L. seedlings, but the fungus also invaded the stele, causing extensive cellular lysis (Fernando and Currah, 1996). The effects of four different strains of *L. orchidicola* were strain- and host-specific, and the symbiotic associations varied from mycorrhizal to parasitic. *Phialocephala fortinii* Wang and Wilcox has an amensal, parasitic or a neutral association with its host, and in combination with *Potentilla fruticosa* L., results in a significant increase in shoot weight (Fernando and Currah, 1996). Root-endophytic *Bacillus* strains possess specific physiological and (or) biochemical characteristics that facilitate colonisation of internal root tissues with subsequent growth-promoting possibilities for the host plant (Shishido et al., 1995).

4.2. Detrimental endophytic associations

4.2.1. Latent pathogenesis

Plant pathologists, rigidly following Koch's postulates, have discarded latent pathogens as "saprophytes" or "secondary parasites", since no symptoms were detected following inoculation of a vigorous host. Alternatively they have labelled latent pathogens as aggressive pathogens without considering possible predisposing factors (Schoeneweiss, 1975).

A parasitic relationship usually starts when the infection hypha of a fungus penetrates the host cuticle and then the outer epidermal cell wall (Verhoef, 1974). In some instances, however,

some time may pass between penetration and the start of such a parasitic relationship, which is then referred to as a latent, dormant, or quiescent infection (Verhoef, 1974). The latent period is defined as the time from infection until the expression of macroscopic symptoms, or as a prolonged incubation period (Sinclair and Cerkauskas, 1996). Only fungi colonising living tissue can potentially be termed latent pathogens (Kowalski and Kehr, 1996). Many pathogens undergo an extensive phase of asymptomatic growth along with colonisation and then latent infection before symptoms appear (Sinclair and Cerkauskas, 1996). Latent-infecting fungi as well as endophytes can infect plant tissues and become established after penetration, but infection does not imply the production of visible disease symptoms. According to Sinclair and Cerkauskas (1996), latent infection of plants by pathogenic fungi is considered one of the highest levels of parasitism. "*Bona fide* endophytism" on the other hand, refers to a latent infection that never results in visible disease symptoms, and a close mutualistic association with the host plant (Sinclair and Cerkauskas, 1996).

Expression of symptoms caused by a latent pathogen can be triggered by two main groups of ellicitors, namely, changes in the host physiology, and environmental stress:

(a) Symptom expression elicited by host physiological changes

Simmons (1963, in Verhoef, 1974) discussed four possible explanations for the latent nature of infections in banana fruit: (i) a toxin may be present in unripe, but not ripe fruit; (ii) the nutritional requirements of the fungus are not met by the composition of green, unripe fruits; (iii) the energy requirements of the fungus are only met when the metabolism of the host change from the unripe to the ripening phase; and (iv) the enzyme potential of the fungus is not strong enough to allow the invasion of the immature fruit, but sufficient to allow the invasion of ripe fruit. Thus, changes in the host physiology of fruits, may trigger disease expression.

Comparative studies done by Espinosa-García and Langenheim (1991) on the effect of essential oils on three pathogenic and one endophytic fungus demonstrated differences in tolerance

to essential oils between pathogens and endophytes. The relatively high tolerance showed by the pathogens, *Phomopsis occulta* (Sacc.) Traverso, *Pestalotiopsis funerea* and *Seiridium juniperi* (Allesch.) Sutton to essential oil phenotypes of redwood, reflect their adaptation to the host defence reactions that involve terpenoids. The coniferous endophyte, *Cryptosporiopsis abietina*, on the other hand, displayed an overall susceptibility to the redwood essential oils (Espinosa-García and Langenheim, 1991).

(b) Symptom expression elicited by environmental stress

A significant number of endophytic fungi in healthy plants become pathogenic when their host plants are weakened. In this instance the host-fungus interaction manifests itself as a disease syndrome (Dorworth and Callan, 1996). These fungi are often referred to as opportunists that may pass from a latent or mutualistic mode to a necrotrophic mode when the host plant is predisposed by several factors such as stress (Schoeneweiss, 1975). This latent phase gives the fungus an advantage over genuine saprophytes in colonising dying branches (Kowalski and Kehr, 1996). There are indications that the natural pruning of tree branches is a process actively enhanced by certain fungi. Kowalski and Kher (1996) thus concluded that several of the fungi isolated from living branch bases are likely to be weak parasites. Their presence in the tissue may, however, prevent colonisation by more aggressive parasites and thus also their spread into the main stem. This gives rise to the possibility that these endophytes may be involved in the natural pruning of stressed tissues (Boddy and Griffith, 1989). Latent infections by endophytes do not result in the formation of disease symptoms, but may weaken the plant, predisposing it to other stresses or diseases (Sinclair and Cerkauskas, 1996).

According to Schoeneweiss (1975), the following factors may act as disease inducing or predisposing factors to change a latent infection by a fungus to a disease syndrome: water stress, which can consist of water deficits and drought, as well as excess water and flooding; temperature

stress, consisting of low temperatures and freezing, as well as high temperatures; defoliation stress; transplanting stress; nutrient stress; and various other factors such as reduced light, toxic substances (herbicides and other pesticides), and wounding which reduce host vigour. Any unusual factor can therefore predispose plants having latent infections resulting in disease symptoms.

Pathogens can survive during latent infection in a quiescent state by adapting either physiologically or morphologically. For example, *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. in Penz., a pathogen of mango and avocado, survives the latent period as dormant appressoria, but persists in blueberry fruit as germinated appressoria and penetration hyphae. In seedling leaves of *Citrus natsudaidai* Hayata it persists as versicolate hyphae in the intercellular spaces of the leaf epidermis. In detached mature citrus fruit latent appressoria and latent hyphae occur within and beneath the cuticle and in the intercellular spaces of the epidermis (Sinclair and Cerkauskas, 1996). Most other latent pathogens survive as inactive, latent hyphae or mycelia within host tissue and intercellular spaces (Sinclair and Cerkauskas, 1996).

Endophytes and weak parasites of *Quercus*, namely *Pezicula cinnamomea* and *Colpoma quercinum*, may contribute to the death of weakened tissue, but the aggressive *Fusicoccum quercus* Oud., causal agent of annual canker, is hardly ever isolated as an endophyte (Kowalski and Kehr, 1996). Latent colonisation of oaks by *Hypoxylon atropunctatum* (Schwein.: Fr.) Cooke, probably accounts for the rapid increase in disease incidence following drought. The greater natural incidence of disease on black oaks compared to white oaks may be related to differences in drought sensitivity (Bassett and Fenn, 1984).

Known pathogens of tropical plants, such as *Colletotrichum* spp., *Fusarium* spp. and *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl., as well as strains of these species, can cause severe damage and losses in forests and plantations (Grey, in Dreyfuss and Petrini, 1984). Their isolation from symptomless plants can be an important aspect generally overlooked in plant

pathology and epidemiology (Petrini and Dreyfuss, 1981, Smith et al., 1996a). Simple mutations could also give rise to pathogenic varieties of endophytes by inducing biotrophic characteristics in certain strains (Boddy and Griffith, 1989).

The causal agent of chestnut blight, *Cryphonectria parasitica*, was isolated as an endophyte by Bissegger and Sieber (1994) from healthy coppice shoots of European chestnut (*Castanea sativa* Mill.). The fungus comprised a small component of all the endophyte assemblages and all *C. parasitica* isolates were of the normal phenotype with a high laccase activity, showing its fitness and potential pathogenicity. Bissegger and Sieber (1994) speculated that the fungus remains latent in the host phellem, until unfavourable conditions such as water stress and wounding of the host lead to the expression of pathogenicity. Three other known pathogens were also isolated from chestnut shoots; *Amphiporthe castanea* (Tul.) Barr, a weak wound parasite causing dieback and canker on *C. sativa*; *Pezicula cinnamomea* (DC.) Sacc., the causal agent of bark cankers on weakened red oak (*Quercus rubra* L.); and *Diplodina castaneae*, which causes "Javart" disease of European chestnut (Bissegger and Sieber, 1994). Smith et al. (1996a) found that the endophytic colonisation of healthy cones of different *Pinus* spp. by the pathogen *Sphaeropsis sapinea* (Fr.: Fr.) Dyko and Sutton, was possitively correlated with the relative susceptibilities of the species to the pathogen. Endophytic colonisation can thus reflect the inherent susceptibilities of different host genotypes.

Kowalski (1993) isolated the pathogen of autumn needle cast, *Cyclaneusma minus* twice as frequently from symptomless needles of trees that showed symptoms of second year needle cast, than from trees without such symptoms. Trees showing needle cast symptoms had an overall higher susceptibility to fungal infection, already on their first year needles (Kowalski, 1993). It is a well-known fact that plant pathogenic fungi express an incubation phase before disease symptoms appear. In the case of *Cy. minus*, this latent phase extends more than 15 months,

which might explain its "endophytic" nature, and high colonisation frequency in pine needles (Kowalski, 1993).

The most frequently isolated endophytic fungi from sessile oak are *Apiognomonia quercina*, *Aureobasidium apocryptum* (Ellis and Everh.) Hermanides-Nijhof and *Colpoma quercinum* (Pers.) Wallr. Although they are reported as weak parasites, they are also present in healthy plant tissue without causing apparent disease symptoms (Halmschlager et al., 1993). *Apiognomonia quercina*, the anamorph of *Discula quercina*, is the causal agent of leaf galls and leaf spots on oak. *Aureobasidium apocryptum* is the causal agent of leaf spots on oak trees, and *Colpoma quercinum* causes infections on twigs and stems of stressed oak trees (Halmschlager et al., 1993). Among the dominant species found on aspen stems and branches, three species with presumed pathogenic abilities were isolated, namely, *Cryptosphaeria populina*, *Cytospora chrysosperma* (Pers.: Fr.) Fr. and *Hypoxylon mammatum* (Wahlenberg) J.H. Miller (Chapela, 1989). *Cryptosphaeria populina* causes bark disease, while *C. chrysosperma* and *H. mammatum* are associated with cankers of aspen (Chapela, 1989).

Cenangium ferruginosum Fr.: Fr. is documented as a pathogen causing shoot dieback of pines, but it also seems to live as an endophyte in the needles of *Pinus sylvestris* (Kowalski, 1993). Wood decay of dying trees possibly originates from infections of latent fungi present in healthy, living branches (Chapela and Boddy, 1988b). These fungi are in a state of physiological dormancy, which is only broken under appropriate environmental conditions which include the reduction of water content in the xylem of the tree. The variation in endophytic colonisation between annual rings could be attributed to variation in tree susceptibility and inoculum potential (Chapela and Boddy, 1988a). *Botryosphaeria dothidea* (Moug.) Ces. Et de Not. is the causal agent of die-back, canker and leaf spots of *Eucalyptus* spp. in South Africa, but it is also able to colonise the xylem and leaves of trees asymptomatically (Smith et al., 1996b). Disease symptoms

develop rapidly at the onset of environmental stress such as frost, hot winds or drought, which can be seen as the trigger for the pathogenic stage of the pathogen (Smith et al., 1996a). Notwithstanding this evidence, the majority of "true" endophytes are not associated with disease symptoms (Boddy and Griffith, 1989). Knowledge of the latent phase of any fungus, the length of the latency and the mechanisms that trigger the fungus to induce symptoms and to reproduce is, however, important for the improvement of disease control measures (Sinclair and Cerkauskas, 1996).

4.2.2 Indirect enhancement of insect colonisation and inhibition of host plant growth

The endophyte, *R. parkeri*, may slightly inhibit the growth of its host, Douglas fir at high levels of infection, but has no other deleterious effect on the growth of the host (Todd, 1988). On the other hand, some endophytes can actually have a positive effect on insect colonisation. Gange (1996) proved that infection of sycamore (*Acer pseudoplatanus* L.) leaves by an endophytic fungus, *Rhytisma acerinum* (Pers.) Fries, positively affected the number of aphids [*Drepanosiphum platanoides* (Schr.) and *Periphyllus acericola* (Walk.)] on leaves, especially during summer. This could possibly be contributed to the higher amount of soluble and total nitrogen, and totalled carbon contents of infected leaves. It is possible that the digestive processes of the fungus alter total carbon or nitrogen contents as compounds are moved into or out of leaves by the host, in this way altering the food quality of these tissues. The presence of endophytes may therefore also determine the seasonal patterns of herbivory by these aphids (Gange, 1996).

4.3. Utilisation and manipulation of endophytic associations

4.3.1. Biocontrol of weeds

Until now the only recognised means of controlling weeds killing or constraining growth of newly

planted forest trees were to use chemical herbicides or by controlled burning. Both of these methods have attracted huge criticism from environmental groups, and thus other means of control have to be investigated (Dorworth and Callan, 1996). Biocontrol agents can be divided in two groups; first-order (I°) biocontrol agents, which can be applied as mycoherbicides for single event weed control or as classical bioagents for continuing weed control, and second-order (II°) biocontrol agents, which are opportunistic weak pathogens (Dorworth and Callan, 1996). First-order (I°) biocontrol, can be defined as: "Direct application of living agents which reduce the individuals of target pest populations either in number or in vigour, or both." Second-order (II°) biocontrol can be defined as: "Manipulation of environmental conditions, the targeted hosts, the indigenous micro flora or all of these in order to induce the natural pathogenicity or stimulate the virulence of the native micro flora, thereby yielding biological control." Some endophytic fungi show promise as II° biocontrol agents of forest weeds, but I° biocontrol does not involve endophytes. Historically, II° biocontrol resorted under categories of crop rotation, mulching and organic amendments, flooding and other techniques. These methods reduce pathogen populations by eliminating nutritional bases, negatively affecting environmental conditions, or by promoting the development of antagonistic micro flora. The same principles can be applied to vegetation management (Dorworth and Callan, 1996). In biocontrol the balance is tipped towards the pathogen, by strengthening the pathogen or weakening the host. Two benefits in the use of indigenous fungi for biocontrol are: operator control, where the operator can limit the reaction by controlling the application of a stress factor quantitatively or qualitatively, and thereby reducing host vigour; and the buffer reaction or natural sink rendered by the natural biosphere. Lack of natural buffering by the biosphere can result in the uncontrolled spread of introduced pathogens as in the case of pine blister rust, chestnut blight and oak wilt (Dorworth and Callan, 1996). Research on biocontrol through the application of endophytes has the goal to promote internal

fungi from resident to necrotrophic status by stimulating the fungi themselves or by reducing the physiologic vigour of the host plant, or reaching a suitable combination of the two. Endophytes themselves may also predispose their hosts to environmental damage by reducing the damage threshold (Dorworth and Callan, 1996).

Manipulation of conditions affecting endophytic fungi in order to utilise their potential as pathogens (as biocontrol agents), should involve manipulation of the host. Two approaches can be considered: the target host plant can be used as a way to translocate agents (chemostimulants) that may stimulate the endophyte into necrotrophic activity, or the target host plant can be subjected to various stress agents, including the application of topical chemicals and physical influences such as heat, cold, drought, etc., which may alter the balance between host and endophyte in the favour of the endophyte (Dorworth and Callan, 1996).

4.3.2. Biocontrol of other pathogens

An endophytic, *Cryptosporiopsis* sp. isolated from *Vaccinium myrtillus* L., produced three different antibiotic-containing substances, which are all inhibitory to *Candida albicans* (C.P. Robin) Berkhout, a common human pathogen (Fisher et al., 1984). Experiments using crude culture filtrate of the fungus indicated antibiotic activity against *Aspergillus niger* Tiegh., *C. albicans*, *Staphylococcus aureus* Rosenbach and *Trichophyton mentagrophytes* (Robin) Blanchard. The continuing needs for less toxic, but more effective drugs which can be administered orally for the treatment of serious *Candida* infections, indicate that further investigations in antibiotic activity such as produced by *Cryptosporiopsis* sp. are required (Fisher et al., 1984). Noble et al. (1991) isolated and identified an echinocandin from an endophytic *Cryptosporiopsis* sp. derived from twigs of *P. sylvestris*, and a *Pezicula* sp. derived from twigs of *Fagus sylvatica*. This compound proved to have antimicrobial properties against certain yeasts. Fungi which produce such potent

antifungal properties give them a competitive advantage over other potential fungal colonisers (Noble et al., 1991).

SUMMARY

The term "endophyte" has evolved to not only describe the location of an organism, but the actual association between the organism and its host plant. True endophytes colonise their host without any symptom expression. They are able to colonise a wide variety of hosts but some endophytic species show strong specificity towards specific host plants. Gymnospermae, which have quite unique types of leaves, harbour their own specialised group of endophytic species.

In order to understand the role of endophytes completely, it is important to study the adaptation of endophytes to their specific environment, as well as the environmental factors that contribute to the different colonisation patterns encountered in the host plant. True endophytes have adapted their infection and colonisation strategies in order to infect and colonise their hosts without causing any deleterious effect on the host nor evoking the defence mechanisms of the host plant. In this way, they exist as biotrophs or may have active mutualistic associations with their particular host plant. Beneficial effects on the host plant may vary from growth enhancement, resistance against disease or attack by insects, to detrimental effects such as indirect enhancement of insect colonisation and disease symptoms such as the case with latent pathogens.

Understanding their adaptation and ecological role in gymnosperms, may lead to the utilisation of endophytes in the holistic management of mixed and monocultural forest ecosystems. They could for example be used as bio-indicators, indicating the effects of air pollution and acid rain. Endophytes can also play an important role in the initial degradation of plant material and debris. Their utilisation as biocontrol agents of weeds or other pathogens, or as protectants against disease and insect infestation, is also documented.

This review has elucidated many of the interactions between endophytes and their gymnosperm hosts. It will hopefully serve as a useful source of information on which to base future research.

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CHAPTER 2

ENDOPHYTIC FUNGI ISOLATED FROM NEEDLES OF THREE *PINUS* SPECIES IN SOUTH AFRICA

ABSTRACT

Pine needles are colonised by various species of endophytic fungi. It is well-known that the endophytic biota varies with host species, as well as with time. The aim of this study was therefore to make a qualitative and quantitative comparison of endophyte populations within the canopies of *Pinus patula*, *P. radiata* and *P. elliottii*, during winter and summer. Pine needles were sampled from plantations in Mpumalanga, South Africa, during July and December. Collections were made from ten trees of each species, four branches from each tree, two from the top of the canopy and two from the bottom. On each branch, five needles were sampled from the base and terminal point, respectively. Two 10 mm pieces from the opposite ends of each needle were surface sterilised and plated onto corn meal agar. This study indicated a significant difference ($P<0.05$) in endophytes numbers between samples collected during winter and summer. *Pinus patula* and *P. elliottii* were most intensively colonised during winter than summer. In *P. radiata*, however, the number of fungi isolated in summer was significantly higher than in winter, but the species composition of fungi remained the same. No significant difference ($P<0.05$) in endophyte colonisation was evident between the top and the bottom of the canopy. However, needle tips were significantly more ($P<0.05$) colonised than needle bases. The most prominent fungi isolated, were *Cyclaneusma minus* and a sterile white yeast-like fungus. *Cyclaneusma minus* is a latent pathogen causing autumn needle cast, and the sterile white yeast-like fungus is suspected to be a true endophyte.

INTRODUCTION

Endophytic fungi are known to have diverse and intricate relationships with their coniferous hosts (Petrini, 1986). In most instances these associations are permanently symptomless and therefore harmless to the host or, in some cases, they can even be beneficial (Carroll, 1988). Under certain environmental and/or physiological conditions however, certain endophytic fungi may express themselves as pathogens (Bissegger and Sieber, 1994). Endophytic fungi can also play a significant role in the successful decomposition of host litter (Sherwood-Pike et al., 1986). The hidden presence of potential pathogens or decomposers in intensively cultivated monocultures of *Pinus* spp. can therefore have important implications for the successful management of such plantations, especially where stress is involved. Smith et al. (1996) have already indicated that latent infections of pine trees by *Sphaeropsis sapinea*, could explain the rapid colonisation of trees after hail damage.

Endophytic fungi have been shown to vary both quantitatively and qualitatively according to host species, age of tissue and season (Carroll and Carroll, 1978). Smith et al. (1996) found a correlation between the susceptibility of specific *Pinus* and *Eucalyptus* species to infection by pathogens, and the frequencies of colonisation by latent pathogens. Seasonal variation is usually linked to the phenology of the host and the seasonal spread of reproductive units of the fungus involved. It has been reported that the species composition of endophytic fungi is usually consistent in successive years, but the frequency of specific fungi may change due to environmental conditions (Hata and Futai, 1996). The species composition and frequency of endophytic fungi will also be influenced by needle senescence and the changing phenology of the host plant (Hata and Futai, 1993), which are in turn influenced by climatic and environmental factors.

Pinus spp. are economically important to the forestry industry in South Africa. A wide variety of pathogenic fungi have a significant impact on the productivity of the industry (Lundquist,

1986, 1987). Many of these may be endophytic. The aim of the present study was to investigate the endophytic fungal population of needles of the three economically most important *Pinus* spp. in South Africa in order to obtain a better understanding of the ecology of foliar fungi in exotic monoculture plantations.

MATERIALS AND METHODS

Pine needles were periodically sampled from pine trees growing in the Mpumalanga province of South Africa. The study area is situated 5 800 m above sea level, 26°15'S, and 30°30'E. Four-year old trees of *P. patula* Schlechtend. & Cham. and *P. elliottii* Engelm. and seven-year old trees of *P. radiata* D. Don. were sampled. Ten trees from each of three respective pine species planted in monoculture stands, at three different sites in close proximity, were sampled during winter (July) and summer (December) 1994. Five needles were sampled from eight positions in each tree (Fig. 1).

A 10-mm-long section of needle was excised from the base and tip of each needle sampled and surface sterilised in ethanol (96%) for 1 min, sodium hypochlorite (5.25%) for 3 min, and ethanol (96%) for 30 sec (see Fig. 1). Sterilised needle sections were subsequently plated onto 1.5% corn meal agar (CMA, Oxoid®, Unipath Ltd, Basingstoke, Hampshire, England), supplemented with 0.1 g/l streptomycin sulphate (Novo Strep®, Novo Nordisk (Pty) Ltd., Johannesburg, South Africa, ai=0.333g/ml) to inhibit bacterial growth. Plates were incubated at room temperature (± 21°C) and inspected at regular intervals for fungal growth. Fungal colonies developing from predetermined batches of needle sections were documented, and subsequently transferred to malt extract agar plates (MEA, 0.5% Difco® Malt extract (Difco Laboratories, Detroit, MI, USA) with 1.5% Oxoid® Agar Technical (Unipath Ltd, Basingstoke, Hampshire, England)) overlaid with sterile (autoclaved) pine needles in order to promote

sporulation and facilitate identification.

Statistical analysis of variance was done with SOLO (BMDP Statistical Software Inc., Los Angeles, CA), using the procedure for a general linear model. Two way ANOVA's were used for analysis of raw data and the Newman-Keuls test for multiple comparison between variables. The overall number of isolates refers to colonisation rates of all infection sites on needles.

RESULTS AND DISCUSSION

The present study confirmed that exotic pine species cultivated in South Africa harbour many diverse fungal species that are presumably endophytic in nature. Qualitative and quantitative differences in fungal colonisation were detected between pine species (Fig. 2), as well as between and within seasons (Fig. 3). Significantly more fungal colonies ($P<0.05$) were isolated from *P. elliottii* and *P. patula* needles in winter than in summer. In contrast, *P. radiata* yielded more fungi in summer than in winter. These findings are consistent with those reported by Hata and Futai (1993) who demonstrated needles of *Pinus densiflora* Siebold and Zucc. to harbour more endophytes during any sampling date than those of *P. thunbergii* Parl.

The high occurrence of *Cyclaneusma minus* in *P. radiata* is primarily responsible for the higher occurrence of endophytes in summer than in winter (Table 1). Since the *P. radiata* plantation was also 3 years older than the *P.patula* and *P. elliottii* plantations, tree age can also be considered as a contributing factor. Endophytes tend to increase in number with increasing needle and tree age (Fisher et al., 1986). These results also possibly reflect differences in host physiology (Hata and Futai, 1993), linked to the fact that *P. radiata* is planted off-site in Mpumalanga. The species is far more suited to winter-rainfall areas of the country, whereas *P. elliottii* and *P.patula* are usually cultivated in the summer-rainfall regions (Poynton, 1979). The greater height of the older *P. radiata* trees, and greater canopy density could also contribute to higher frequencies of

endophytes (Carroll, 1995).

Our study showed that for both P. elliottii and P. radiata, the bottom part of the canopy yielded a significantly higher ($P<0.05$) colonisation percentage, than the top part (Fig. 4). According to Johnson and Whitney (1989) this can probably be attributed to the downward movement of water-borne fungal propagules in the canopy, with no preference for any particular side of the crown. North and south location of foliage sampled had no significant effect on the frequency of fungi isolated (Fig. 5). This is consistent with results obtained by Johnson and Whitney (1989). Needles on the outside of the canopy were significantly less ($P<0.05$) colonised than needles on the inside of the canopy (Fig. 6). Both these tendencies can probably be attributed to the greater amount of spores available for infection in the lower inside part of the canopy. The same tendency was found by Petrini and Carroll (1981) in a study of endophytic fungi in foliage of Cupressaceae from the Oregon region (USA). In general, infection and colonisation of endophytes is influenced by the microclimate that exists within the tree canopy (Todd, 1988). The higher frequency of endophytes on the inside of the canopy could also be attributed to needle age, since needles on the ends of branches are usually younger than those in the inner region. This phenomenon is consistent with findings of endophytes in *Ulex europaeus* and *U. gallii* by Fisher, Anson and Petrini (1986).

Needle tips were significantly more ($P>0.05$) heavily colonised than the basal parts excluding the fascicle (Fig. 7). This is in contrast to observations made of endophytes in *Betula pubescens* var. *tortuosa* (Helander et al., 1993). It is probably due to the base of the needle being protected by the fascicle, and generally, less exposed to inoculum than the needle tip.

Differences between *Pinus* species in terms of fungal taxa were detected primarily in the less frequently isolated fungi, but also to a lesser extent in the more frequently isolated species (eg. *Fusarium subglutinans* in *P. elliottii* and *Cyclaneusma minus* in *P. radiata,* Table 1). An

unidentified sterile white, yeast-like fungus contributed to the highest percentage of colonies isolated from *P. patula* (in winter) and *P. elliottii* (winter). The high occurrence of *Cyclaneusma minus* (syn. *Naemacyclus minor*), causal agent of autumn needle cast (Crous et al., 1990), could be explained by its long incubation period of 10-15 months (Kistler and Merrill, 1978; Kowalski, 1988). This would furthermore explain its lower frequency in winter, when infection is still taking place, than in summer, when the infection process has been completed, just 3 months before symptoms of needle cast will occur. *Sphaeropsis sapinea* was isolated from healthy *P. radiata* needles during the winter. This important needle pathogen of pines (Crous et al., 1990) was also isolated by Smith et al. (1996) from healthy pine cones and is also considered to be a latent pathogen.

According to Petrini (1986, 1996), specificity of endophytes can usually be determined by their frequencies, where frequently encountered endophyte species are species specific, and less frequent endophytes non-specific. *Aureobasidium pullulans* was isolated from all three pine species, at significantly lower frequencies than *C. minus* and the sterile white yeast-like fungus, which suggests that it is a non-specific endophyte or a host neutral endophyte (Boddy and Griffith, 1989). *Aureobasidium pullulans* has been described as part of the epiflora of coniferous needles (Carroll et al., 1977), but also a genuine endophyte (Pugh and Buckley, 1971). Endophytes such as *Epicoccum purpurascens* and *Fusarium subglutinans* were only isolated from *P. elliottii*, and in larger frequencies than many other endophytic species. *Epicoccum purpurascens* is regarded as a non-specific endophyte and is isolated from a variety of hosts (Petrini, 1986). *Fusarium subglutinans*, however, could be a host specific endophyte, but in general the frequency of endophytic colonisation of *P. elliottii* needles was too low to give decisive explanations for its endophytic species composition.

The role of foliar endophytes in the ecology of pine trees can probably be explained by a

closer examination of the taxa of endophytic fungi isolated. According to Millar (1974) several fungi are involved in the microbial succession of decomposing coniferous leaf litter. These include *Aureobasidium pullulans, Lophodermium pinastri, Naemacyclus* sp. (syn. *Cyclaneusma*), *Chaetomium* spp. and *Cladosporium*, which we isolated as putative endophytes (Table 1). Early infection of needles could thus give these fungi ample time to be the first colonisers of shed needles.

Host-endophyte relationships in *Pinus* spp. and the factors influencing them are complicated, and will require more intensive studies to fully address all the issues discussed above. General information about the distribution of foliar endophytes throughout seasons and within the canopy, as well as the distribution within needles, can assist foresters in managing monoculture plantations. The occurrence of different latent pathogens that have an endophytic growth phase will have important implications for disease management in plantations. The role of endophytes in litter decomposition is also relevant to general plantation management, and should not be underestimated.

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Table 2. Frequencies of endophytic species isolated from pine needles.

	Fungal colonies as percentage of colonies isolated per host species					
	Winter			Summer		
Fungus	*P. patula*	*P. elliottii*	*P. radiata*	*P. patula*	*P. elliottii*	*P. radiata*
Aureobasidium pullulans (de Bary) G. Arnaud	0.7	1.0	0.9	5.5		1.3
Nigrospora oryzae (Berk & Boome) Petch.	1.4	2.8	0.6		14.9	
Chaetomium Kunze: Fr. sp.	0.7		0.3			0.2
Nodulisporium G. Preuss. sp.		0.5	0.1			
Xylaria hypoxylon (L.) Grev.	0.7		0.1			
Cyclaneusma minus (Butin) Di Cosmo, Peredo & Minter	7.1		36.1	12.7		56.0
Fusarium subglutinans (Wollenweb. & Reinking) P. E. Nelson, T. A. Toussoun & Marasas		0.5			29.7	
Alternaria tenuiissima (Kunze: Fr.) Wiltshire	1.4	2.0				
Sporormiella Ellis & Everh. spp.	9.3	5.7	0.5		14.9	0.4
Sphaeropsis sapinea (Fr.) Dyko & Sutton			0.1			
Acremonium Link:Fr. spp.				3.6	8.1	
Coniochaeta (Sacc.) Cooke. sp.	0.7		0.1			
Epiccocum purpurascens Ehrenb.		0.5			8.1	
Ascochyta Lib. sp.		0.5				
Drechslera Ito. sp.	0.7					
Lophodermium Chev. sp.		2.5	0.3			0.2
Gonytrichum Nees & T. Nees sp.			0.1			
Eustilbum Rabenh. sp.			0.1			
Cladosporium Link:Fr. sp.			0.3			0.4
Phomopsis (Sacc.) Bubák. sp.				3.6		
Gremmeniella M. Morelet sp.						0.2
Yeast spp.	8.5	2.8	14.5	18.2	4.0	16.1
Sterile white yeast-like fungus	48.3	43.7	31.5	9.0		18.0
Sterile white and black fungus	2.1	1.5	0.1		8.1	1.3
Sterile grey white fungus	0.7	0.9	0.1	5.5		0.2
Sterile brown fungus	1.4	2.8	2.5	7.3	2.7	1.6
Sterile cream tufty fungus	1.4	10.9	1.7	9.1	5.4	1.3
Sterile black-brown fungus	3.5	3.8	1.0	18.2	1.4	0.8
Sterile pink fungus		11.4	1.7		2.7	1.2
Black yeast like fungus	1.4	0.9	0.5	5.5		0.3
Sterile white wooly fungus		1.9	0.5	1.8		0.5
Sterile dark pink fungus		0.5	0.1			

Figure 1. A schematic representation of the sampling and surface sterilisation procedures of pine needles for the recovery of endophytes.

OUTSIDE OF CANOPY		INSIDE OF CANOPY	
Bottom	North facing (b1)	Bottom	North facing (b2)
	South facing (b4)		South facing (b3)
Top	North facing (t1)	Top	North facing (t2)
	South facing (t4)		South facing (t3)

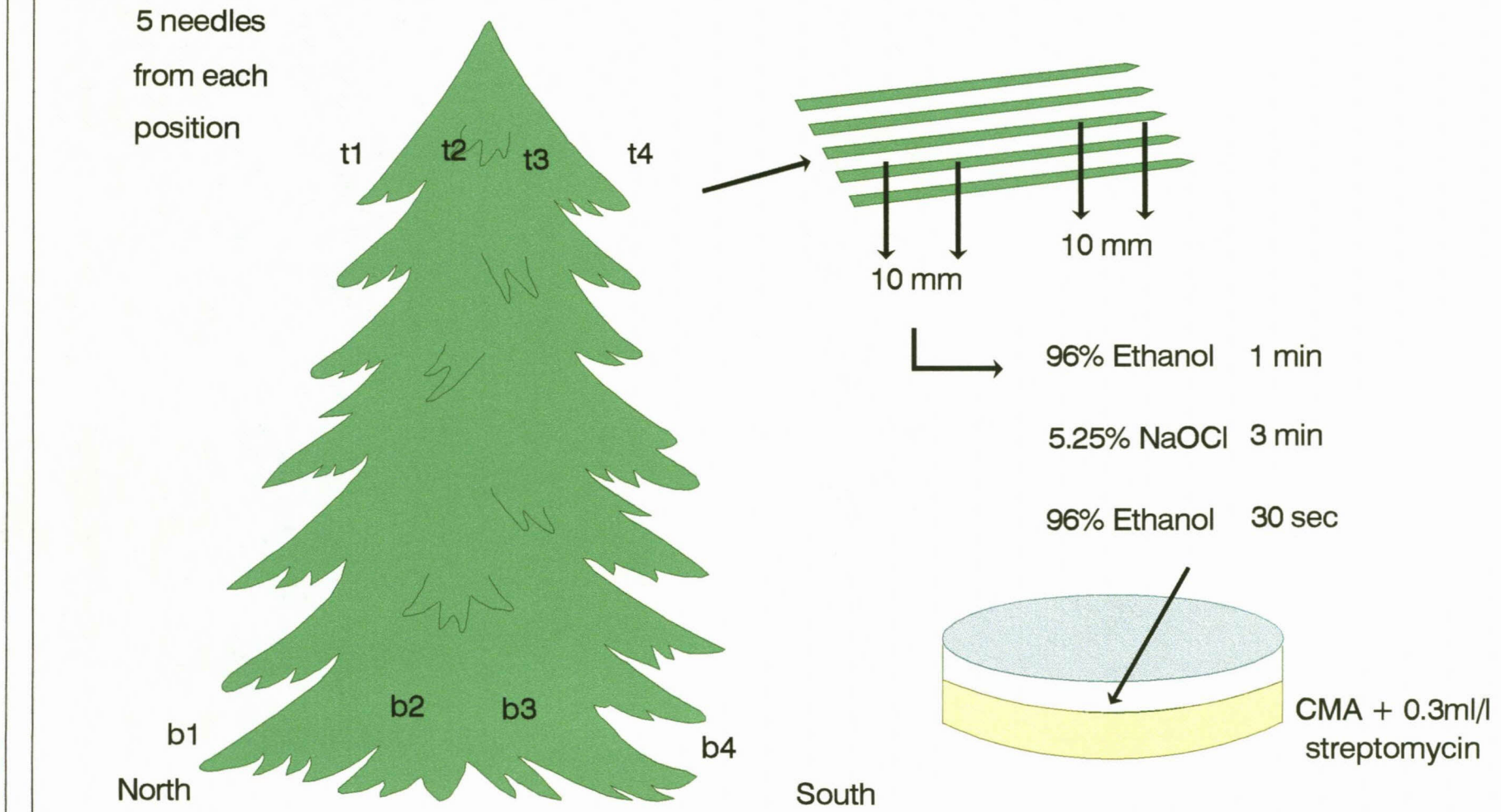
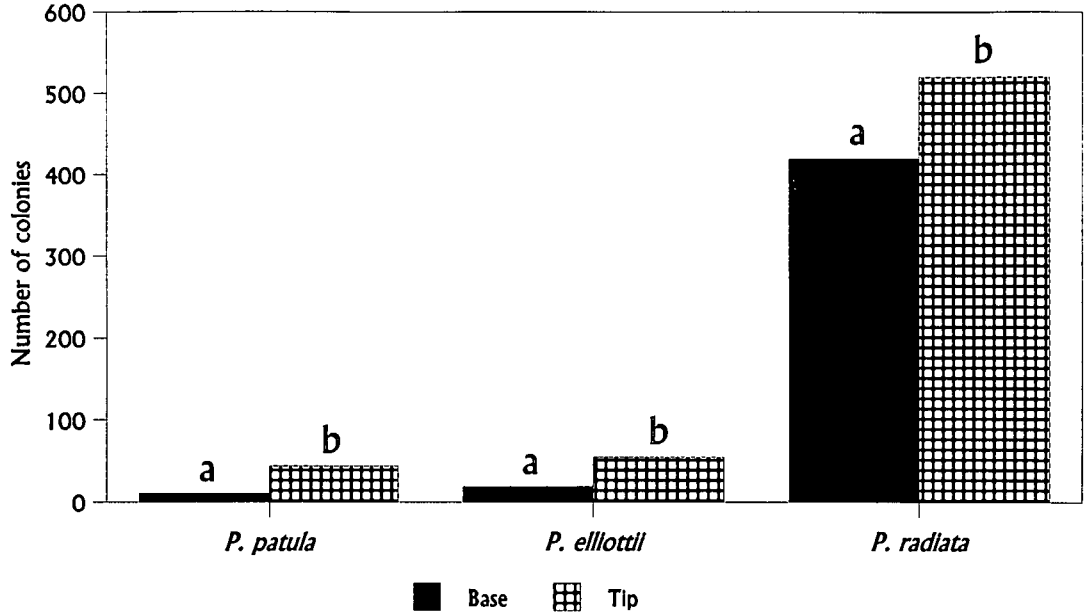

5 needles
from each
position
t1
t2
t3
t4
b1
b2
b3
b4
North
South
10 mm
10 mm
96% Ethanol 1 min
5.25% NaOCl 3 min
96% Ethanol 30 sec
CMA + 0.3ml/l
streptomycin

Figure 2. **Differences between species, according to position on needle blade.** Bars denoted by lower case letters that differ for each species, indicate significant differences at $P < 0.05$ according to the Newman-Keuls test.

Figure 3. **Seasonal differences in endophytic colonisation of needles from three *Pinus* species.** Bars denoted by lower case letters that differ for each species, indicate significant differences at $P < 0.05$ according to the Newman-Keuls test.


600
500
400
300
200
100
0
Number of colonies
a
b
a
b
a
b
P. patula
P. elliottii
P. radiata
Base
Tip

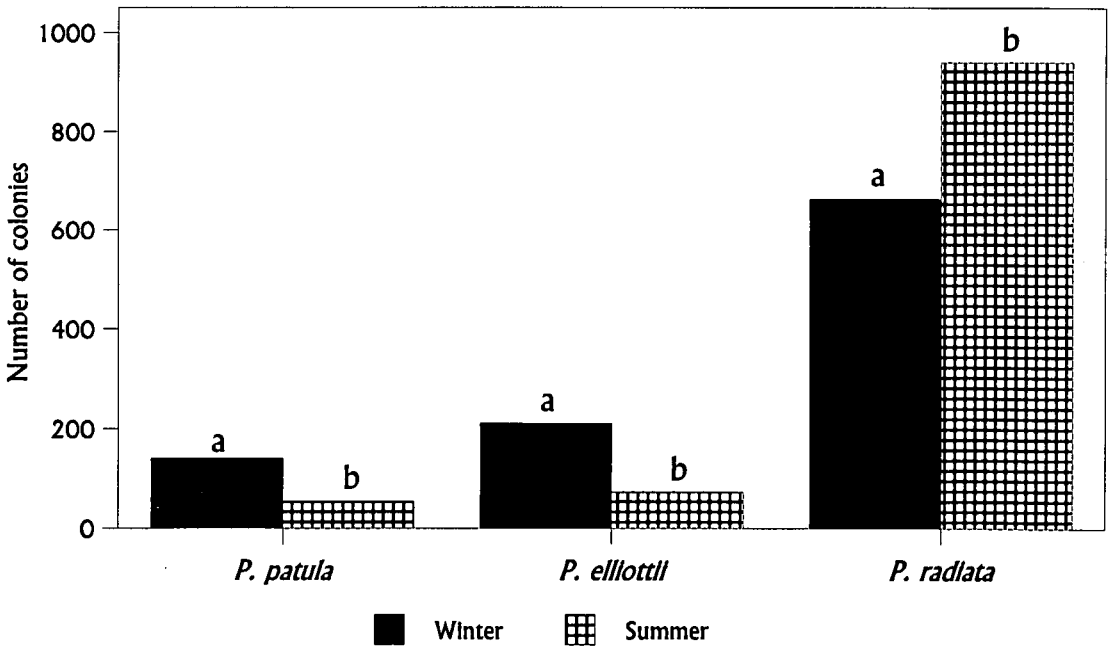
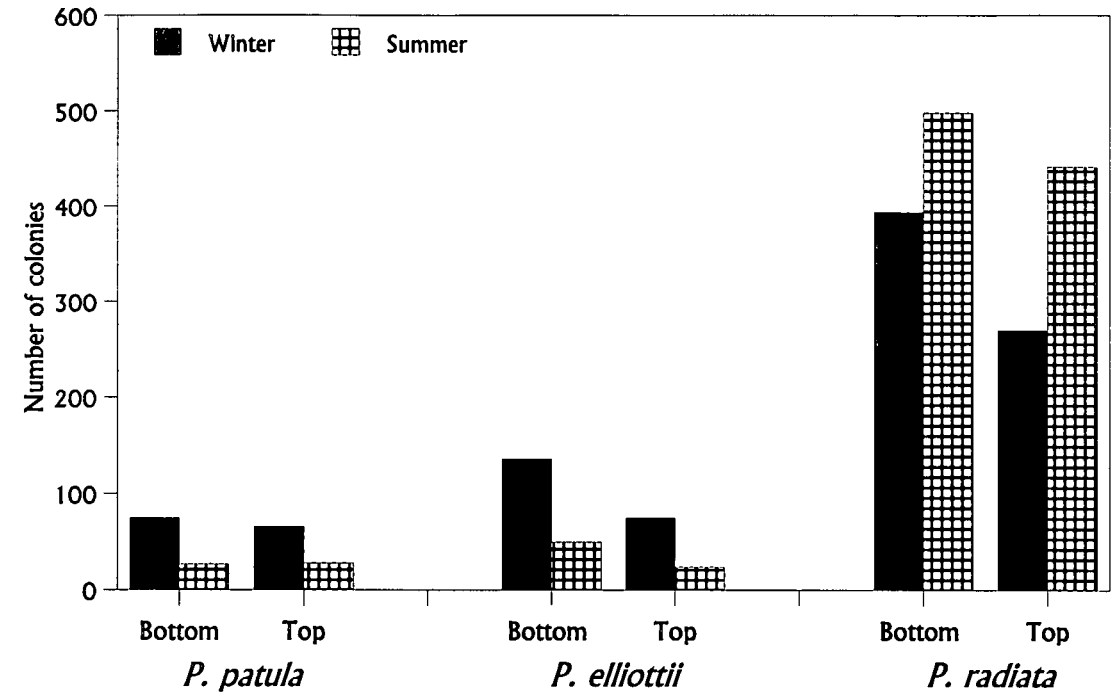

1000
800
600
400
200
0
Number of colonies
a
b
a
b
a
b
P. patula
P. elliottii
P. radiata
Winter
Summer

Figure 4. Differences in colonisation frequencies between the bottom and top of the canopies of the three *Pinus* spp.

Figure 5. Differences in colonisation frequencies between the northern and southern parts of the canopies of the three *Pinus* spp.


600
500
400
300
200
100
0
Number of colonies
Winter
Summer
Bottom
Top
Bottom
Top
Bottom
Top
P. patula
P. elliottii
P. radiata

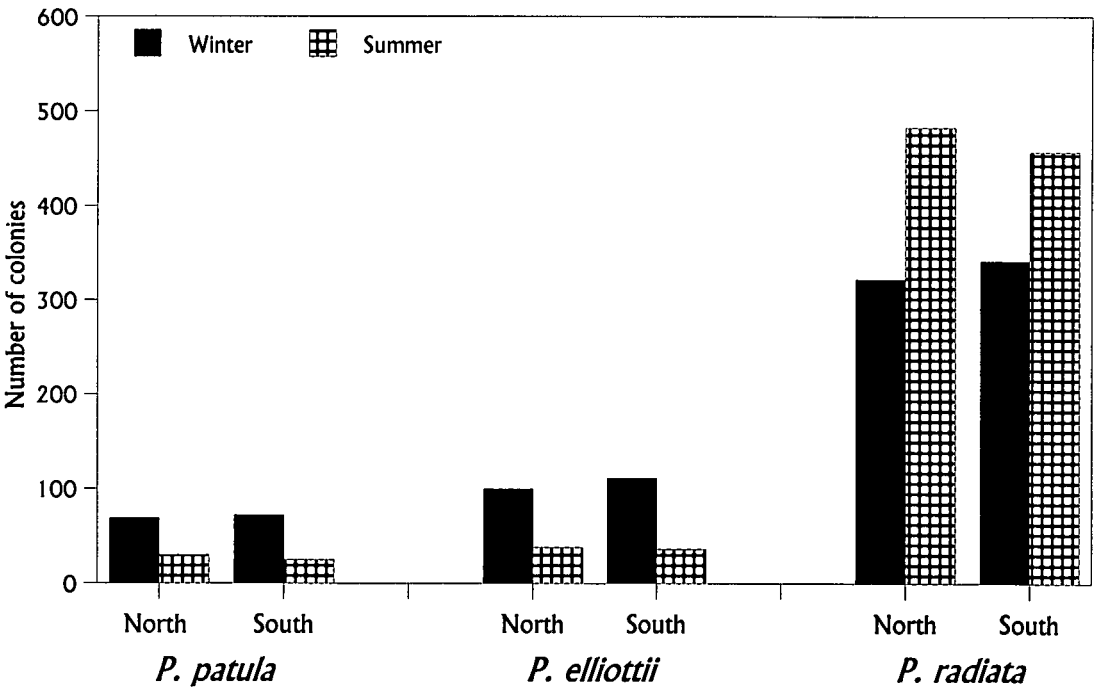
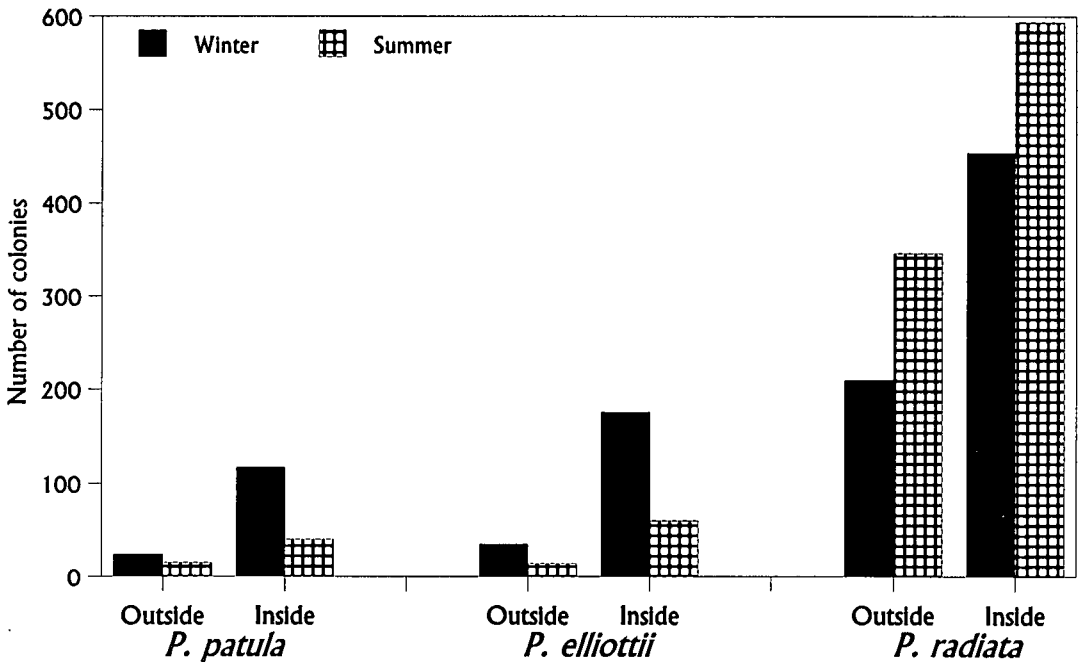

600
500
400
300
200
100
0
Number of colonies
Winter
Summer
North
South
North
South
North
South
P. patula
P. elliottii
P. radiata

Figure 6. Differences in colonisation frequencies between the outside and inside of the canopies of the three *Pinus* spp.


600
500
400
300
200
100
0
Number of colonies
Winter
Summer
Outside
Inside
Outside
Inside
Outside
Inside
P. patula
P. elliottii
P. radiata

CHAPTER 3

DISTRIBUTION OF FUNGAL ENDOPHYTES IN NEEDLES OF *PINUS RADIATA*.

ABSTRACT

Endophytic fungi are known to display specificity towards host plant tissue. The aim of this study was therefore to determine the distribution of fungal endophytes in *Pinus radiata*. Needle samples were taken during four seasons from two eight-year-old *P. radiata* trees; one separate, solitary tree and one growing in an even-aged, plantation nearby. Five needle fascicles of four different age groups were collected from each tree. One needle per fascicle was surface sterilised, cut into 12 equal sections and plated onto 1.5 % corn meal agar containing ciclosporin (5 mg/l) and streptomycin (0.3 ml/l). Plates were incubated at 5°C for at least three months before fungal colonies from each needle section were counted and identified. Plates were subsequently incubated at 21°C for a further month to ensure that all fungi had emerged at 5°C. In general, fewer endophytes were isolated from the solitary tree than the plantation tree. Qualitative and quantitative differences in endophyte populations were observed within needles as well as between needle age groups and seasons.

INTRODUCTION

At the most basic level, the term "endophyte" would include all organisms that live inside a plant (Petrini, 1986). These endophytic organisms can range from a wide variety of fungi (Petrini, 1986) to several bacterial species (Chanway, 1996). In the broader sense, the term has evolved to include latent pathogens and symptomless endophytes. However, when the biology of endophytism is viewed in the strict sense, it becomes apparent that only endophytes which do not induce symptoms in their hosts, can be considered true endophytes (Wilson, 1995). A problem with this definition

arises however, when isolations are made from symptomless plant material, and latent pathogens are recovered. Further research may shed some light on aspects of latent pathogenicity, quiescent infection and possible predisposing factors that may lead to the expression of disease symptoms (Schoeneweiss, 1975). It is therefore important that suitable methods are used for isolating endophytes in order to ensure that all epiphytes are excluded. Care should be taken to ensure that plant material is processed as soon as possible, to prevent any epiphytes from entering through stomata, and that very intensive sterilisation methods are used to superficially sterilise plant material (Millar and Richards, 1974).

Plant/endophyte interactions may vary from mutualistic, antagonistic, to neutrally symbiotic (Carroll, 1988). Studies of tree endophytes are important, since some branch, leaf, and trunk endophytes may provide similar benefits to plants that mycorrhizae do (Kowalski and Kehr, 1996). Foliar endophytes may provide protection against insect herbivory (Clark et al., 1989; Calhoun et al., 1992) or serve as antagonists against other pathogens (Carroll, 1988). Endophytes can also act as bio-indicators of host vitality by showing the effects of acid rain, ozone depletion and industrial emissions (Helander et al., 1993, 1996), and as bioregulators, by inducing resistance to disease (Bissegger and Sieber, 1994). Since colonisation of host cells by endophytes usually increases during needle senescence, endophytes are usually the first colonisers of shed foliage (Sherwood-Pike et al., 1986). This would suggest they also play an important role in the decomposition of leaf litter (Millar, 1974), by gaining an early advantage over other soil inhabiting decomposers (Rodriguez and Redman, 1997).

The colonisation of plant tissue by endophytic fungi displays very distinct patterns within the host, which are influenced by a variety of factors. Endophytes show specificity towards certain host tissue and organs as well as displaying a degree of ontogenetic specificity (for example needle petioles are more heavily colonised than the needle apex and older needles are more heavily colonised

than young ones) (Sieber-Canavesi and Sieber, 1987). The diversity of endophytic fungal species and their colonisation patterns in pine needles should therefore be studied in the context of: the number of species per sample; distribution within samples; age of needles; the total number of organisms per sample; and the time of year that sampling takes place (Miller, 1995). The aim of the present study was to investigate the colonisation of endophytic fungi in two *Pinus radiata* D. Don. trees of a similar age, growing in close proximity, but influenced by different microclimates.

MATERIALS AND METHODS

Needle samples were taken during four seasons from two eight-year-old *P. radiata* trees, one solitary and one growing 4.65 km away in an even-aged monoculture plantation in Mpumalanga Province, South Africa. The study area is situated 5 800 m above sea level, and 26°15'S; 30°30'E. Both trees originated from the same seedlot and were planted at approximately the same time. Soil and rainfall conditions at the two sites were similar during the entire study period. The only difference was that the solitary tree was never pruned, whereas the plantation tree was pruned at 3 and 7 years. Sampling was performed on autumn equinox (21 March, 1996), winter solstice (21 June, 1996), spring equinox (22 September, 1996), and summer solstice (21 December, 1996) (Preston-Whyte and Tyson, 1988).

Asymptomatic needles were collected from each of four branches per tree (top and bottom, facing north and south). Three to four age groups of needles (depending on the availability) and five needle fascicles per age group were sampled on each branch. Needles were packed in marked bags and kept at 1-5°C until they could be processed in the laboratory within 48 hours of collection. This procedure ensured that epiphytes did not enter the needle tissue and establish themselves in the epicuticular layer (Millar and Richards, 1974).

One needle was removed from each fascicle and sterilised while still attached to the fascicle

sheath. The fascicle sheath was retained on the first needle section (1), to compare the fungi to those in the needle blade. Surface sterilisation was performed by immersing needles in a series of 96% ethanol (1 min), 5.25% sodium hypochlorite (3 min), and 96% ethanol (30 sec). Needles were subsequently cut into 12 equal sections (each approximately 3-10 mm long, depending on the needle age) and plated in chronological order, onto square bio-assay dishes (245 x 245 x 25 mm) (©A/S Nunc, Roskilde, Denmark) containing 1.7% corn meal agar (Oxoid®, Unipath Ltd, Basingstoke, Hampshire, England). Ciclosporin (Sandimmun®, Sandoz, Switzerland, ai = 50mg/ml) at 5 mg/l (Rodrigues, 1994) was added to the medium to retard growth of fungi, and streptomycin (Novo Strep®, Novo Nordisk (Pty) Ltd., Johannesburg, South Africa, ai = 0.333g/ml) at 0.3 ml/l was added to discourage the growth of bacteria (Figs. 1 and 2). Plates were incubated for a minimum of 3 months at 5°C, to give developing colonies sufficient time to emerge. All colonies growing from each needle section were counted and representative re-isolations were made from groups similar in appearance. Plates were then incubated for a further month at 21°C to establish if all the fungi did emerge at 5°C. Additional taxa were noted and identified. For identification purposes fungi were re-isolated onto half-strength malt extract agar (0.5% malt extract and 2% agar technical, Difco®, Difco Laboratories, Detroit, MI, USA,), overlaid with sterile (autoclaved) *P. radiata* needles to promote sporulation. No artificial light sources were used, as cultures were incubated on open laboratory benches at room temperature ($\pm$21°C).

Raw data were analysed using the SAS General Linear Models (GLM) Procedure and differences between means were determined using SAS Duncan's Multiple Range Test (SAS Institute Inc., 1988).

RESULTS AND DISCUSSION

A significant number and variety of fungi grew from pine needle sections. Significant differences ($P < 0.05$) were observed between the four seasons (Fig. 3), with the lowest frequencies observed

from needles collected in spring and winter. Higher frequencies observed in summer and autumn could possibly be attributed to the phenology of *P. radiata*, which has two growth flushes, the major growth flush occurring during spring (Whitehead et al., 1994). A secondary flush occurs during autumn, but considerably less growth takes place as needles are shorter (66 mm) compared to those produced during spring (94-136 mm) (Whitehead et al., 1994). This would explain the lower frequencies of endophytes in newly emerged spring growth needles. Lower numbers of endophytes in the winter are presumably also due to newly emerged autumn needles. Since less new growth occurs during autumn than spring, this would explain the lower numbers of endophytic fungi isolated during spring than in winter needles. The autumn growth flush did not effect frequencies in needles sampled during autumn, since the collection of material took place before the growth flush.

Seasonal differences in endophyte frequencies could also be attributed to the succession of fungi in needles and the increasing colonisation of older needles (Hata and Futai, 1993; Hata et al., 1998). Significant differences ($P < 0.05$) observed between the four age groups, with oldest needles yielding the highest frequency of endophytes (Fig. 2), are consistent with findings of Petrini and Carroll (1981), Todd (1988) and Carroll (1995). Magan et al. (1995) examined the endophytic flora in needles of Sitka spruce (*Picea sitchensis* L.). Endophytic fungi were isolated from all three age classes of needles, with the predominant species being *Rhizosphaera kalkhoffi* Bubak. Results showed colonisation to increase with needle age, although the mean level of colonisation remained less than 5% (Magan et al., 1995). Endophytic flora of sessile oak (*Quercus petraea*) showed seasonal and geographical variations. Leaves were most frequently colonised by *Aureobasidium apocryptum* (48.2%) and *Apiognomonia quercina* (24.9%), with distinct differences noted for various leaf portions (Halmschlager et al., 1993). Fungi such as *Aureobasidium apocryptum*, *Cladosporium cladosporioides* and *Alternaria alternata* were most commonly isolated in September (autumn). *Verticicladium trifidum* was restricted to the May (Spring) sampling, while *Apiognomonia*

quercina and *Aureobasidium apocryptum* were most frequently isolated in July (summer) (Halmschlager et al., 1993).

Host vigour plays an important role in the colonisation of plant organs by fungi, with stressed plants generally being more susceptible to fungal infections (Schoeneweiss, 1975). Density of host plants influences the extent of colonisation in specific plants (Kowalski and Kehr, 1996). It may be assumed that the solitary tree was growing more vigorously than the plantation tree, which was probably stressed due to competition for light, nutrients and moisture. One of the most important factors influencing the environmental adaptation of *P. radiata* in a specific area, is photosynthetically active radiation (Grey, 1989). *Pinus radiata* is consequently suitable for planting between 30° and 46° latitude only. Energy conversion of radiation is also effected by the leaf area index (Grey, 1989). Stress of the plantation tree would thus result due to off-site planting (26°15'S) and increased competition for radiation. The lower leaf area index of the plantation tree due to the fact that is was pruned twice (3 and 7 years) and had a smaller canopy than the unpruned solitary tree would exacerbate stress. According to Hinze and Van Laar (1986), pruning of *P. radiata* increases mortality, especially in older trees. Since stress favours the infection of plants by facultative parasites (Schoeneweiss, 1975) and many fungi isolated in the present study are probably also facultative parasites, it would explain why the plantation tree yielded more fungi.

In the context of endophytic life styles, latent pathogens would relate to facultative parasites and true endophytes to obligate parasites (Rodriguez and Redman, 1997). Thus, higher frequencies of true endophytes can be expected in vigorously growing trees, and latent pathogens in stressed trees. This tendency is reflected in the qualitative and quantitative differences in endophytic colonisation in the two trees studied (Figs. 3 and 4). The plantation tree showed significantly ($P < 0.05$) higher frequencies of *Cyclaneusma minus*, a latent pathogen (Kistler and Merrill, 1978; Kowalski, 1988, 1993), than the solitary tree, which harboured a high frequency of the sterile white yeast-like fungus,

suspected of being a true endophyte. This phenomenon suggests that qualitative and quantitative data relating to endophytic population of pines and other plants could possibly be indicative of host vitality. Although in contrast to the present results, Swart et al. (1998) found that *Amaranthus hybridus* displayed higher frequencies of endophytes in non-stressed plants compared to plants submitted to nutrition and water stress.

The differences observed in the frequency of endophyte colonisation within the respective canopies of the two trees sampled, are consistent with a study conducted by Legault et al. (1989). In the latter study, closed canopies of *Pinus banksiana* and *P. resinosa* showed higher frequencies of colonisation than open canopies. Differences in microclimate, light intensity, and other environmental factors such as temperature and humidity could explain similar frequencies in the top and bottom of the solitary tree canopy, and the top of the plantation tree. This is in direct contrast to the bottom canopy of the plantation tree which showed a higher percentage of other fungi but a lower percentage of sterile white yeast-like fungus (Figs. 5-8). This is most probably due to the downward movement of fungal propagules, deposited in the bottom of the canopy by gravity and rain (Johnson and Whitney, 1989), and the effect of a different microclimate. The density of the canopy in the bottom part of the solitary tree is similar to that at the top of both trees. The openness of the canopy could explain differences in colonisation frequencies between the bottom portion of the canopies of the two trees. *Cyclaneusma minus* had a lower colonisation frequency in the bottom portion of the plantation tree's canopy (Figs. 7 and 8). This may be due to premature abscission of colonised or parasitised needles (Sherwood and Carroll, 1974). Leaf senescence may also trigger increased endophytic colonisation (Sherwood-Pike et al., 1986). Alternatively, premature senescence of needles may be a result of endophytic colonisation which could be the case with *C. minus*, a latent pathogen causing autumn needle cast (Kistler and Merrill, 1978; Kowalski, 1988, 1993).

According to Kowalski (1993), pine needles show the highest frequency of endophytic

colonisation in the middle section of the needles. Bernstein and Carroll (1977) revealed higher colonisation at the needle tip than at the base of the needles. Our results revealed that the needle section together with the fascicle sheath (1), harbours a relatively high number of endophytes, of which the majority were not encountered in successive needle sections. This is due to the difference in chemical composition with the fascicle sheath consisting of more phenols and cellulose than the rest of the needle blade (Carroll and Carroll, 1978; Carroll and Petrini, 1983; Hata and Futai, 1995). The next three sections (2-4) yielded significantly ($P<0.05$) less endophytes than the rest of the needle blade (Fig. 9). This can possibly be explained by the growth of the needle, where sections closest to the fascicle sheath would have been exposed to inoculum for a shorter period of time. This part of the needle is covered by the fascicle sheath during the early stages of growth, but the sheath retracts as the needle grow older. It is interesting to note that the needle sections with the fascicle sheath in the solitary tree had a far higher colonisation frequency than those of the plantation tree. This is probably due to a proportionally larger volume of tissue observed in the fascicle sheath on needles of the solitary tree.

The role of the fungus we refer to as "sterile white yeast-like fungus" in pine needles, is not clear. The fungus could not be identified to date, but would seem to be a specialised endophyte, due to its high frequency of occurrence and its substrate utilisation abilities (See Chapter 4). *Cyclaneusma minus*, causal agent of autumn needle cast, on the other hand, is a latent pathogen, with a long asymptomatic incubation period of 10-15 months (Kistler and Merrill, 1978; Kowalski, 1988, 1993).

The results of our study indicate definite seasonal differences in endophytic colonisation of pine needles as well as other differences relating to endophytic colonisation between two trees growing under different ecological conditions. Management practices in plantations that reduce or increase the environmental stress on individual trees, thereby influencing endophytic populations,

could have definite beneficial or negative effects on the general vitality of trees. The most important effects of certain practices would be the influence they have on latent pathogens such as *C. minus*. Other endophytes may have positive effects on the growth of trees. They may have direct physiological effects on vigour (Fernando and Currah, 1996) or indirectly, by acting as biological control agents of pathogens (Stovall and Clay, 1991; Bissegger and Sieber, 1994; Wilson, 1996;) and herbivorous insects (Todd, 1988; Clark et al., 1989; Calhoun et al., 1992). Further investigation of foliar endophytic fungi of intensively managed pine plantations is therefore justified with a view to understanding the effects management practices have on their ecology.

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Figure 1. A schematic representation of the sampling technique used to sample needles from two *P. radiata* trees.

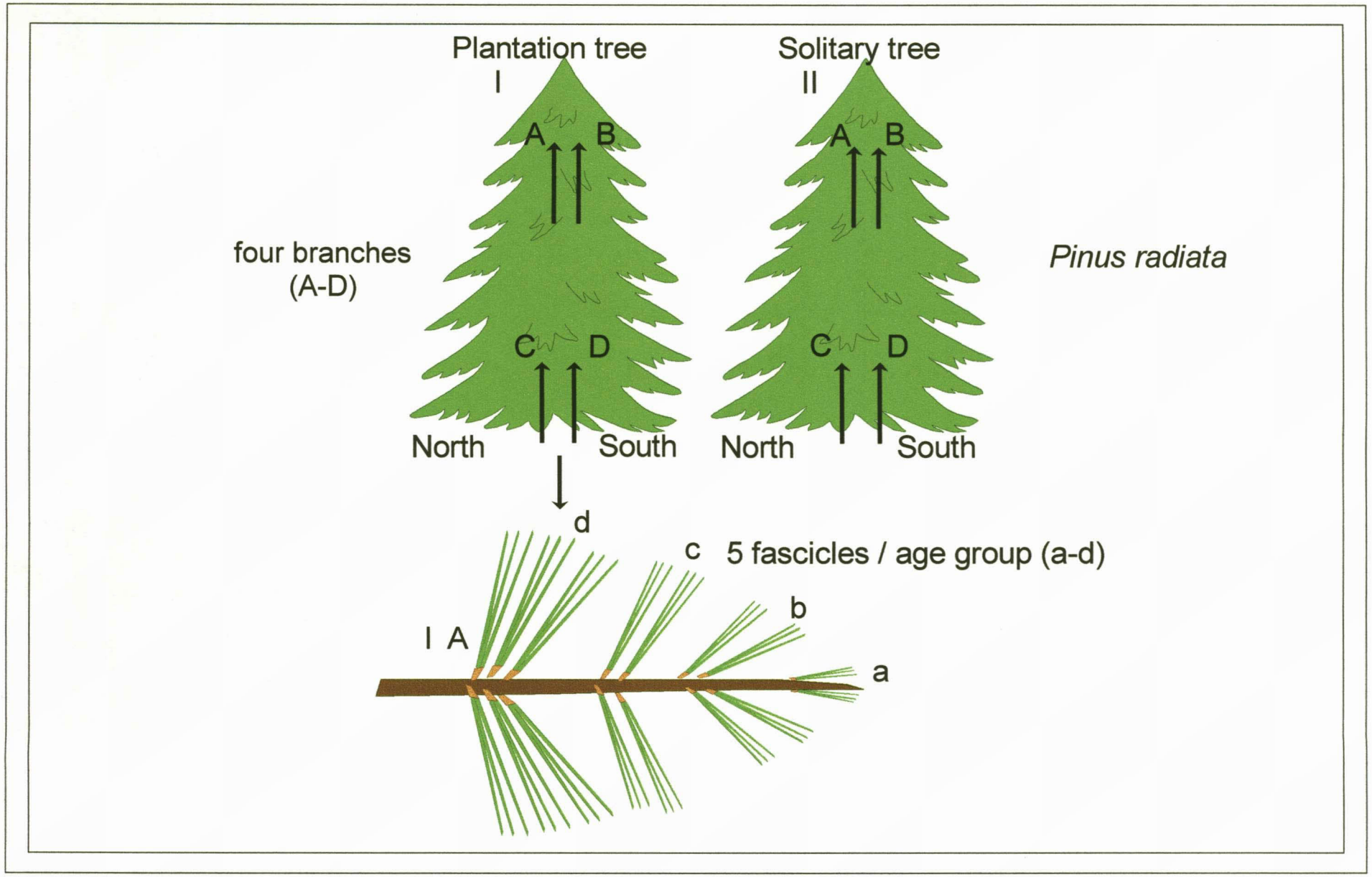

Plantation tree
I
Solitary tree
II
A
B
C
D
four branches
(A-D)
North
South
North
South
Pinus radiata
d
c
5 fascicles / age group (a-d)
b
I A
a

Figure 2. A schematic representation of the isolation process used to recover endophytes from needles of *P. radiata*. In this figure, IAa1 represents: 1-plantation tree; A-top, north facing branch; a-youngest age group; 1-one of 5 needles.

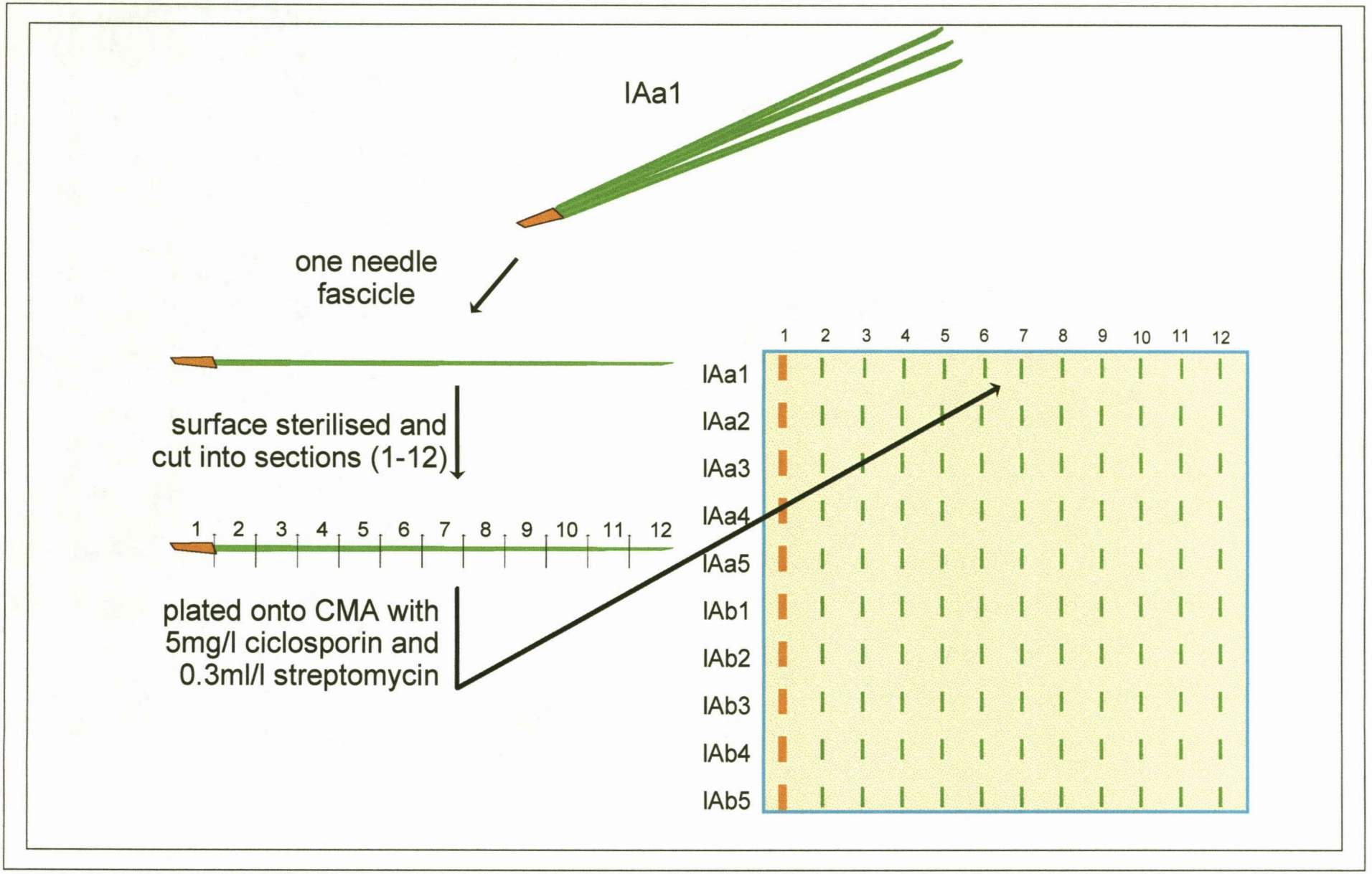
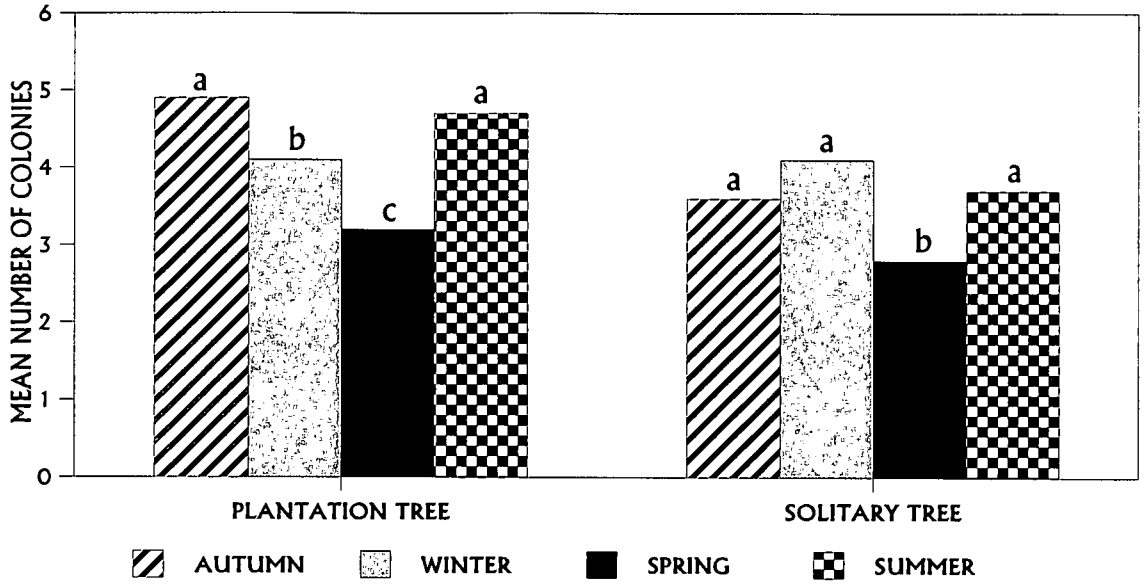

IAa1
one needle fascicle
surface sterilised and cut into sections (1-12)
1 2 3 4 5 6 7 8 9 10 11 12
plated onto CMA with 5mg/l ciclosporin and 0.3ml/l streptomycin
1 2 3 4 5 6 7 8 9 10 11 12
IAa1
IAa2
IAa3
IAa4
IAa5
IAb1
IAb2
IAb3
IAb4
IAb5

Figure 3. Mean number of endophytic colonies isolated during the different seasons, from a solitary and plantation tree, respectively. [Bars denoted by the same alphabetical letter for each respective sampling unit, are not significantly different ($P < 0.05$) according to Duncan's test (SAS Institute Inc., 1988)].

Figure 4. Mean number of endophytic colonies isolated from needles of different age groups from a solitary and plantation tree, respectively. [Bars denoted by the same alphabetical letter for each respective sampling unit are not significantly different ($P < 0.05$) according to Duncan's test (SAS Institute Inc., 1988)].


MEAN NUMBER OF COLONIES
6
5
4
3
2
1
0
a
b
c
a
a
a
b
a
PLANTATION TREE
SOLITARY TREE
AUTUMN
WINTER
SPRING
SUMMER

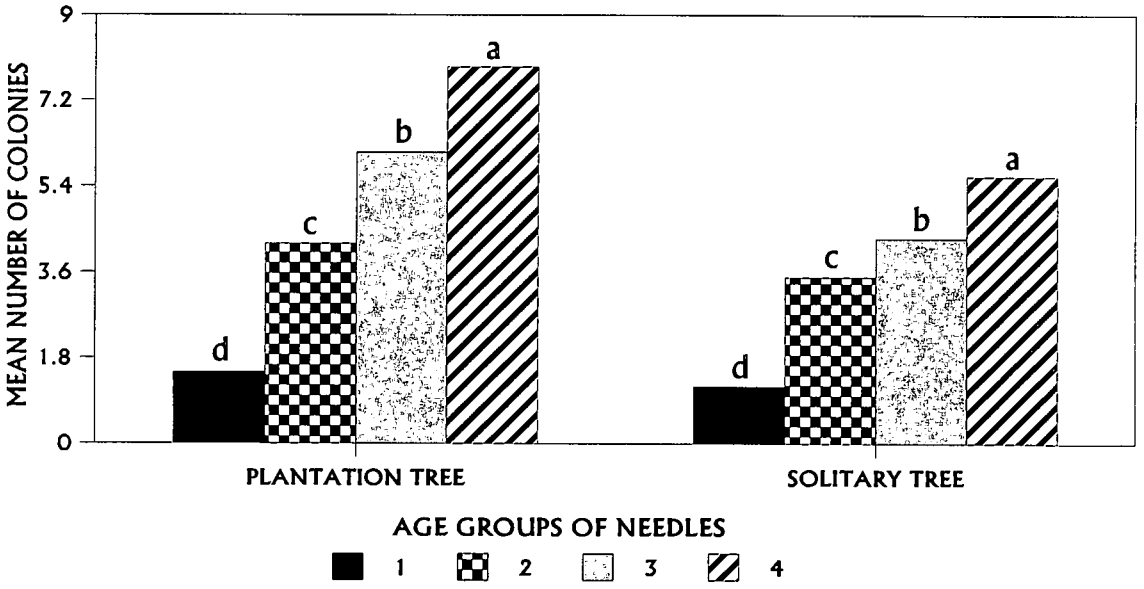
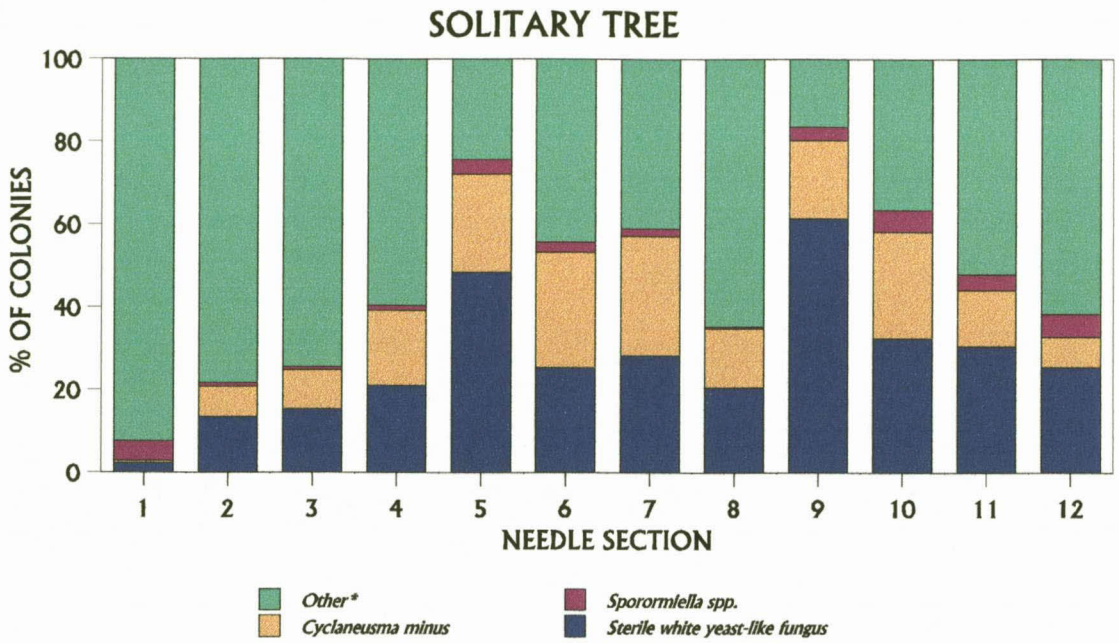

MEAN NUMBER OF COLONIES
9
7.2
5.4
3.6
1.8
0
d
c
b
a
d
c
b
a
PLANTATION TREE
SOLITARY TREE
AGE GROUPS OF NEEDLES
1
2
3
4

Figure 5. Biogeographical distribution of endophytic fungal species in needles of a solitary *P. radiata* tree.

* Other fungi include the following:		
Alternaria tenuissima (Kunze: Fr.) Wiltshire	*Chaetomium* Kunze: Fr. sp. 1	Yeast spp.
Pestalotiopsis funerea (Desmaz.) Steyaert.	*Chaetomium* Kunze: Fr. sp. 2	Basidiomycete 1
Pestalotiopsis Steyaert. sp.	*Epicoccum purpurescens* Ehrenb.	Basidiomycete 2
Xylaria J. Hill ex Schrank spp.	*Cladosporium* Link: Fr. sp.	Sterile yellow yeast-like fungus
Nigrospora oryzae (Berk & Boome) Petch.	*Gilmaniella* Barron. sp.	Sterile white wooly fungus
Nodulisporium G. Preuss. sp.	Hyphomycete 1	Sterile white and black fungus

Figure 6. Biogeographical distribution of endophytic fungal species in needles of a *P. radiata* tree growing in a plantation.

* Other fungi include the following:		
Alternaria tenuissima (Kunze: Fr.) Wiltshire	*Chaetomium* Kunze: Fr. sp. 1	Yeast spp.
Pestalotiopsis funerea (Desmaz.) Steyaert.	*Chaetomium* Kunze: Fr. sp. 2	Basidiomycete 1
Pestalotiopsis Steyaert. sp.	*Epicoccum purpurescens* Ehrenb.	Basidiomycete 2
Xylaria J. Hill ex Schrank spp.	*Cladosporium* Link: Fr. sp.	Sterile yellow yeast-like fungus
Nigrospora oryzae (Berk & Boome) Petch.	*Gilmaniella* Barron. sp.	Sterile white wooly fungus
Nodulisporium G. Preuss. sp.	Hyphomycete 1	Sterile white and black fungus


SOLITARY TREE
% OF COLONIES
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12
NEEDLE SECTION
Other*
Cyclaneusma minus
Sporormiella spp.
Sterile white yeast-like fungus

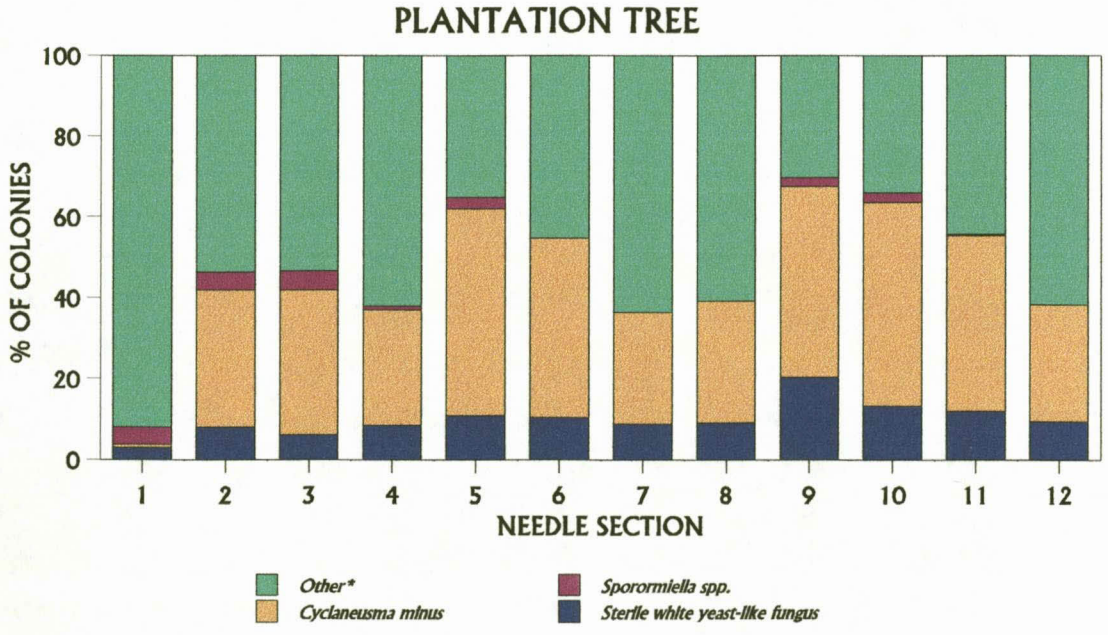
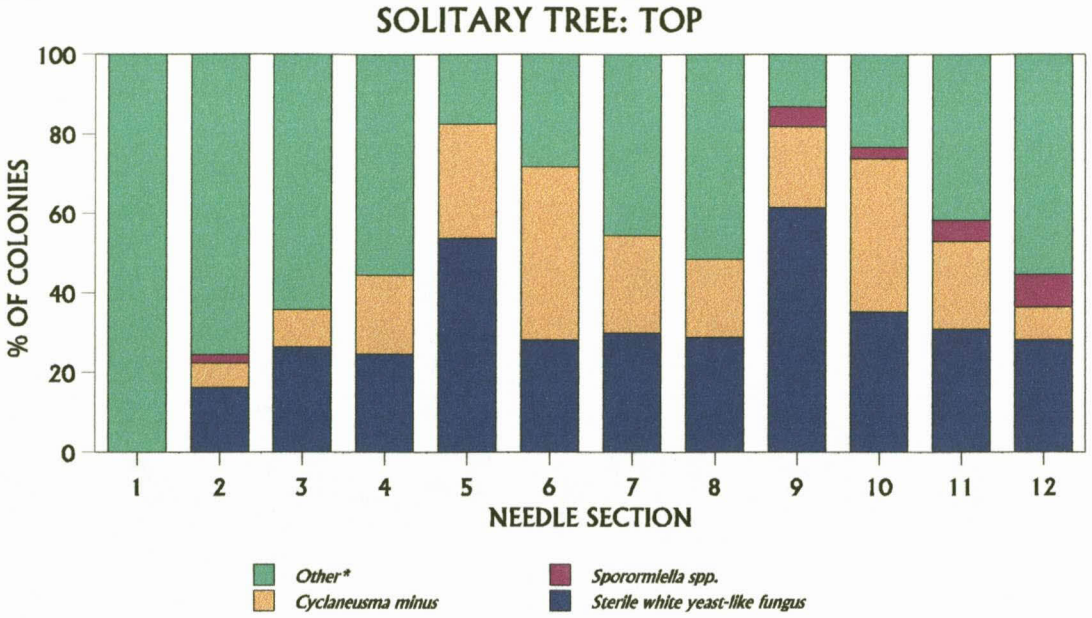

PLANTATION TREE
% OF COLONIES
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12
NEEDLE SECTION
Other*
Cyclaneusma minus
Sporormiella spp.
Sterile white yeast-like fungus

Figure 7. Biogeographical distribution of endophytic fungal species throughout needles collected from the top part of the canopy of a solitary *P. radiata* tree.

* Other fungi include the following:		
Alternaria tenuissima (Kunze: Fr.) Wiltshire	*Chaetomium* Kunze: Fr. sp. 1	Yeast spp.
Pestalotiopsis funerea (Desmaz.) Steyaert.	*Chaetomium* Kunze: Fr. sp. 2	Basidiomycete 1
Pestalotiopsis Steyaert. sp.	*Epicoccum purpurescens* Ehrenb.	Basidiomycete 2
Xylaria J. Hill ex Schrank spp.	*Cladosporium* Link: Fr. sp.	Sterile yellow yeast-like fungus
Nigrospora oryzae (Berk & Boome) Petch.	*Gilmaniella* Barron. sp.	Sterile white wooly fungus
Nodulisporium G. Preuss. sp.	Hyphomycete 1	Sterile white and black fungus

Figure 8. Biogeographical distribution of endophytic fungal species throughout needles collected from the bottom part of the canopy of a solitary *P. radiata* tree.

* Other fungi include the following:		
Alternaria tenuissima (Kunze: Fr.) Wiltshire	*Chaetomium* Kunze: Fr. sp. 1	Yeast spp.
Pestalotiopsis funerea (Desmaz.) Steyaert.	*Chaetomium* Kunze: Fr. sp. 2	Basidiomycete 1
Pestalotiopsis Steyaert. sp.	*Epicoccum purpurescens* Ehrenb.	Basidiomycete 2
Xylaria J. Hill ex Schrank spp.	*Cladosporium* Link: Fr. sp.	Sterile yellow yeast-like fungus
Nigrospora oryzae (Berk & Boome) Petch.	*Gilmaniella* Barron. sp.	Sterile white wooly fungus
Nodulisporium G. Preuss. sp.	Hyphomycete 1	Sterile white and black fungus


SOLITARY TREE: TOP
% OF COLONIES
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12
NEEDLE SECTION
Other*
Cyclaneusma minus
Sporormiella spp.
Sterile white yeast-like fungus

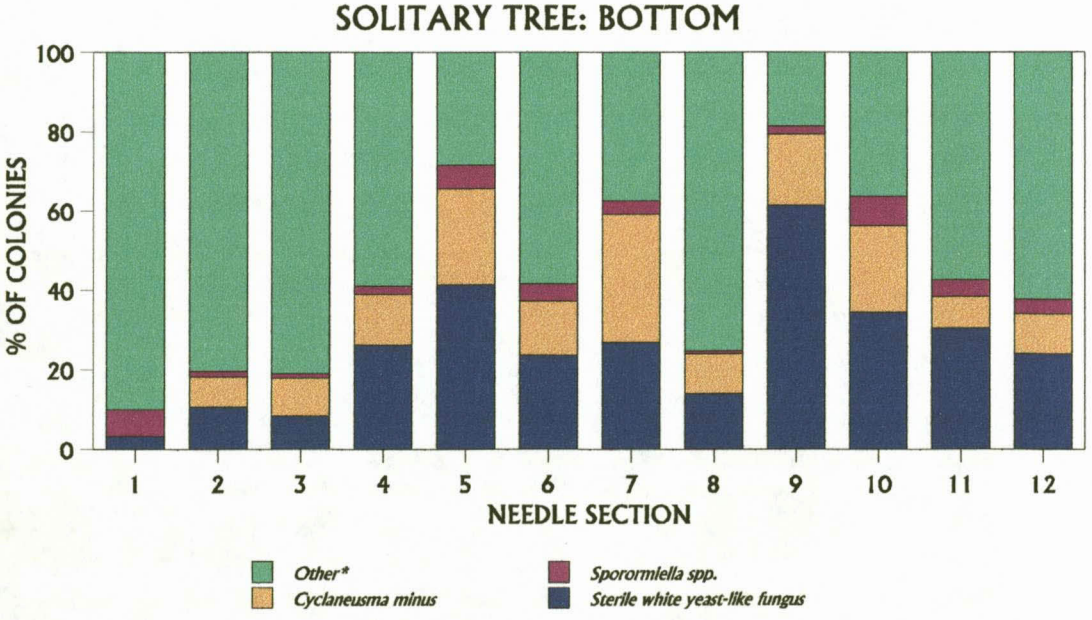
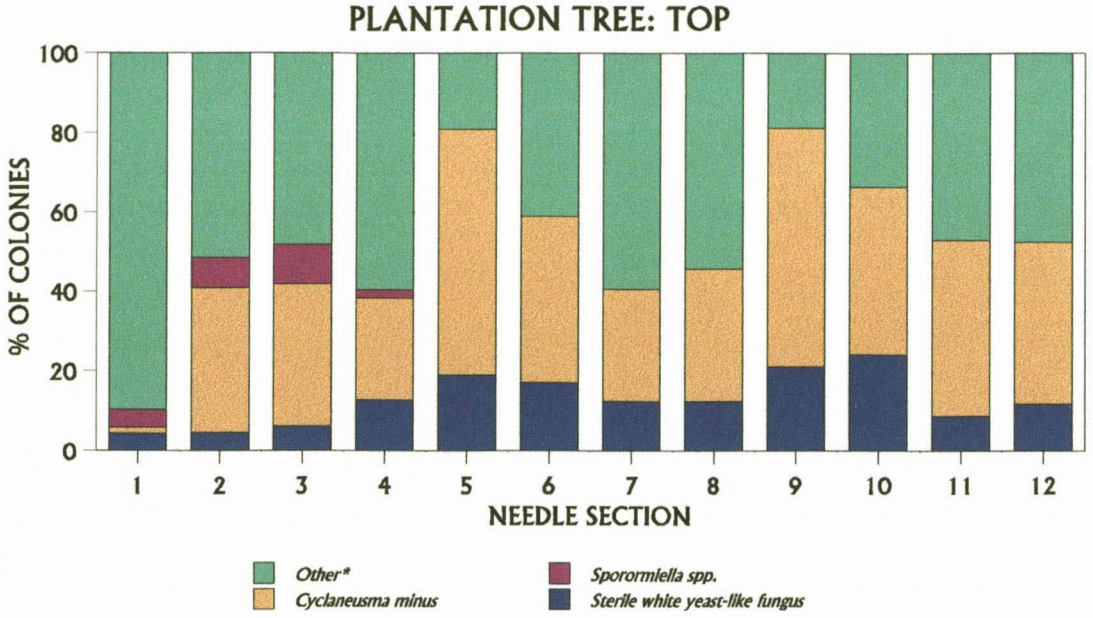

SOLITARY TREE: BOTTOM
% OF COLONIES
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12
NEEDLE SECTION
Other*
Cyclaneusma minus
Sporormiella spp.
Sterile white yeast-like fungus

Figure 9. Biogeographical distribution of endophytic fungal species throughout needles collected from the top part of the canopy of a *P. radiata* tree growing in a plantation.

* Other fungi include the following:		
Alternaria tenuissima (Kunze: Fr.) Wiltshire	*Chaetomium* Kunze: Fr. sp. 1	Yeast spp.
Pestalotiopsis funerea (Desmaz.) Steyaert.	*Chaetomium* Kunze: Fr. sp. 2	Basidiomycete 1
Pestalotiopsis Steyaert. sp.	*Epicoccum purpurescens* Ehrenb.	Basidiomycete 2
Xylaria J. Hill ex Schrank spp.	*Cladosporium* Link: Fr. sp.	Sterile yellow yeast-like fungus
Nigrospora oryzae (Berk & Boome) Petch.	*Gilmaniella* Barron. sp.	Sterile white wooly fungus
Nodulisporium G. Preuss. sp.	Hyphomycete 1	Sterile white and black fungus

Figure 10. Biogeographical distribution of endophytic fungal species throughout needles collected from the bottom part of the canopy of a *P. radiata* tree growing in a plantation.

* Other fungi include the following:		
Alternaria tenuissima (Kunze: Fr.) Wiltshire	*Chaetomium* Kunze: Fr. sp. 1	Yeast spp.
Pestalotiopsis funerea (Desmaz.) Steyaert.	*Chaetomium* Kunze: Fr. sp. 2	Basidiomycete 1
Pestalotiopsis Steyaert. sp.	*Epicoccum purpurescens* Ehrenb.	Basidiomycete 2
Xylaria J. Hill ex Schrank spp.	*Cladosporium* Link: Fr. sp.	Sterile yellow yeast-like fungus
Nigrospora oryzae (Berk & Boome) Petch.	*Gilmaniella* Barron. sp.	Sterile white wooly fungus
Nodulisporium G. Preuss. sp.	Hyphomycete 1	Sterile white and black fungus


PLANTATION TREE: TOP
% OF COLONIES
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12
NEEDLE SECTION
Other*
Cyclaneusma minus
Sporormiella spp.
Sterile white yeast-like fungus

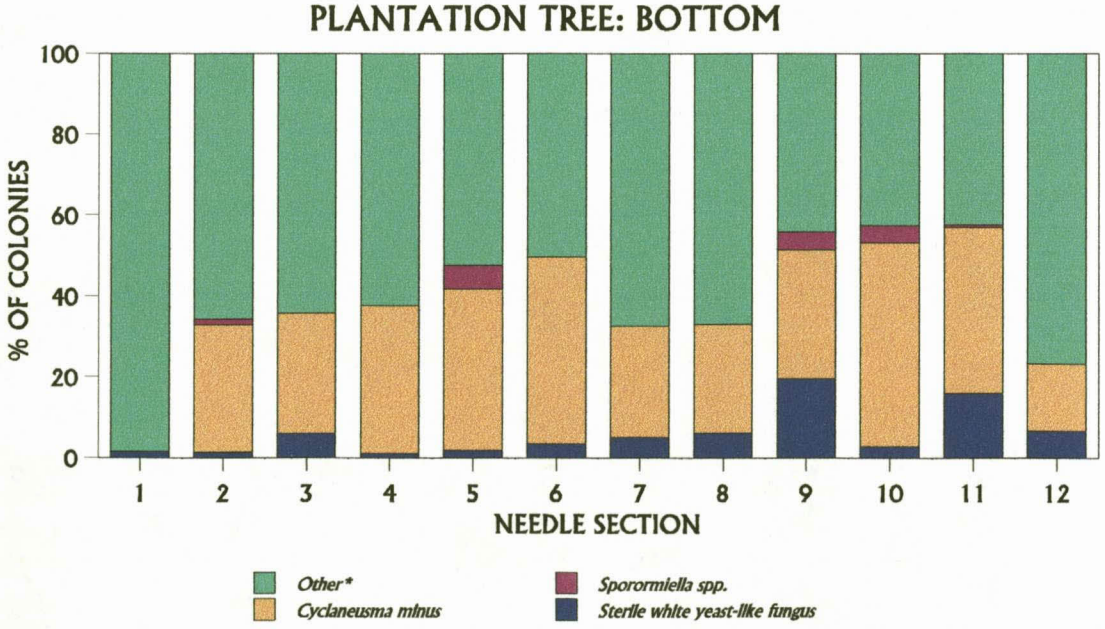
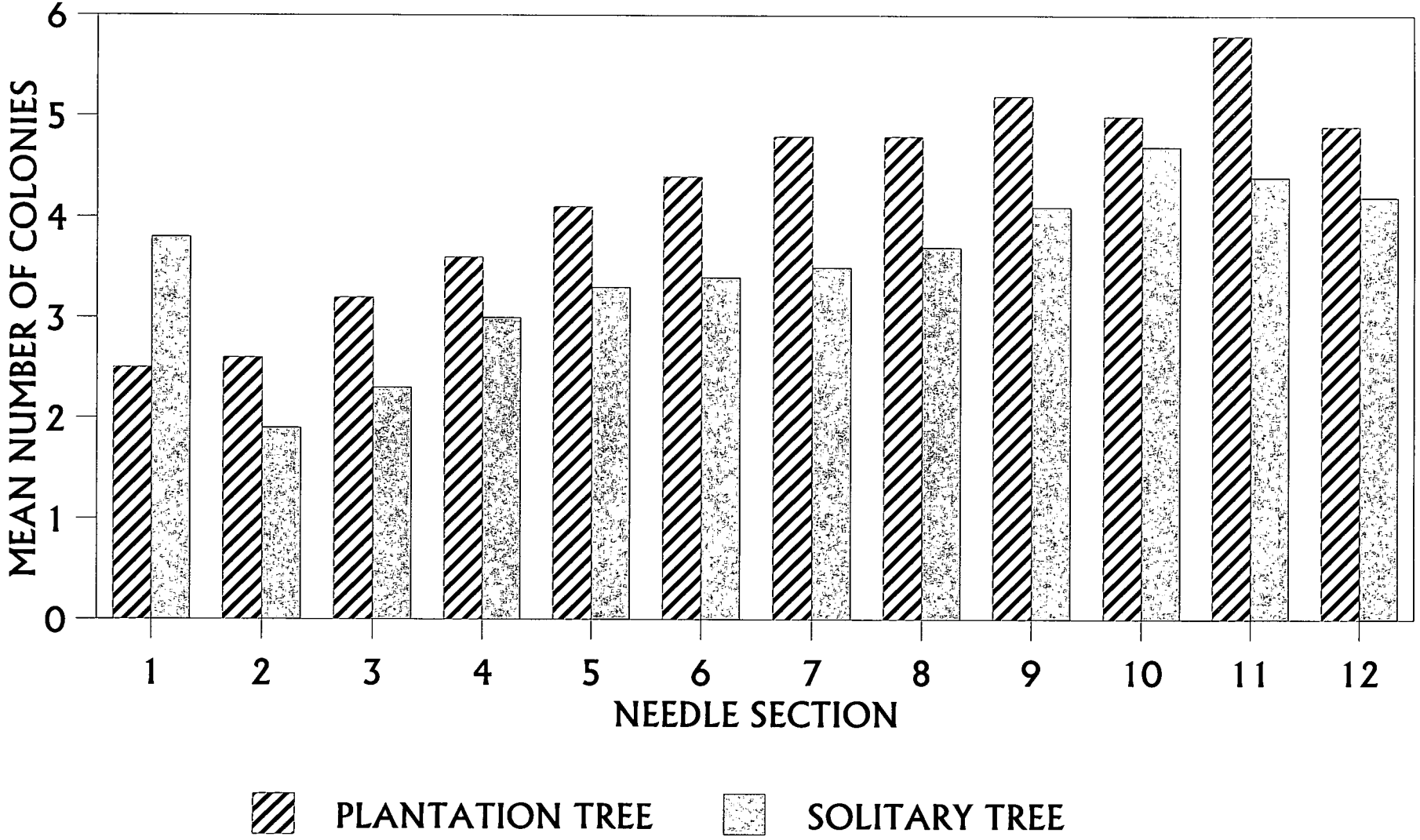

PLANTATION TREE: BOTTOM
% OF COLONIES
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12
NEEDLE SECTION
Other*
Cyclaneusma minus
Sporormiella spp.
Sterile white yeast-like fungus

Figure 11. Mean quantitative distribution of endophytic fungal colonies throughout the needle sections of a solitary *P. radiata* tree and one growing in a plantation.


MEAN NUMBER OF COLONIES
0
1
2
3
4
5
6
1
2
3
4
5
6
7
8
9
10
11
12
NEEDLE SECTION
PLANTATION TREE
SOLITARY TREE

CHAPTER 4

ENZYME PRODUCTION BY ENDOPHYTES ISOLATED FROM PINE NEEDLES

ABSTRACT

True endophytic fungi do not cause symptoms in their plant hosts. However, to enter host tissue they would require mechanisms that would include the production of extracellular enzymes. The aim of this study was to conduct a qualitative assay of enzyme production of 21 predominant fungal endophytes isolated from pine needles. The most important enzymes involved in the infection and colonisation of plants by pathogenic fungi were assayed. They included cellulase, pectinase, lipase, laccase, phenol oxidase, protease, ß-glucosidase, cytochrome oxidase, and peroxidase. Results were consistent with attributes associated with leaf penetration and long-term residence of fungi within pine needles. All fungi screened produced at least two of the enzymes assayed. Different substrate utilisation patterns suggest biochemical partitioning of nutritional resources by endophytes. The ubiquitous presence of lipolytic activity in all isolates tested, suggests the ability to lyse cuticular waxes in order for penetration to occur. The tolerance of most tested fungi to tannic acid (phenol oxydase production) suggests low sensitivity to phenolic compounds (tannins etc.) normally present in pine needle tissue.

INTRODUCTION

Endophytes are organisms that form symptomless infections in plant hosts (Petrini, 1986). Endophytic fungi can form close physiological associations with a wide variety of plant hosts. Dating from when the first observations of endophytes were made by Neill in 1939, endophytic fungi have been isolated from various coniferous plants (Carroll et al., 1977; Carroll and Carroll, 1978; Petrini and Müller, 1979; Petrini and Carroll, 1981; Petrini, 1986; Suske and

Acker,1987; Hata and Futai, 1996), grasses (Clay, 1988), Ericaceaous plants (Petrini, 1984), tropical plants (Dreyfuss and Petrini, 1984), wheat seeds (Sieber, 1989), subalpine plants (Fernando and Currah, 1996), arctic plants (Fisher et al., 1995), several broad leafed hosts (Petrini et al., 1982; Halmschlager et al., 1993; Wilson and Carroll, 1994; Dobranic et al., 1995) and palms (Rodrigues, 1994).

In each of the above instances, endophytes were isolated from different plant tissues and organs. Endophytes display great specificity towards the specific plant substrate that they colonise and many show biogeographical distribution patterns within their hosts, especially in coniferous hosts (Bernstein and Carroll, 1977; Carroll and Carroll, 1978; Petrini and Carroll, 1981; Espinosa-Garcia and Langenheim, 1990; Dobranic et al., 1995). Certain endophytes of *Alnus* spp. colonise only bark, while others colonise only the xylem (Fisher and Petrini, 1990). Certain endophytes only occur in young stems (Barklund and Kowalski, 1996) and others exclusively in the branch bases of forest tree species (Kowalski and Kehr, 1992). Several endophytic fungi have also been isolated from non-mycorrhizal roots (Holdenrieder and Sieber, 1992; Ahlich and Sieber, 1996).

Rodriguez and Redman (1997) designated four classes of endophytic fungi: (1) fungi that actively grow through host tissues, resulting in extensive colonisation; (2) fungi that actively grow through host tissues but only colonise a small percentage of host tissues; (3) fungi that are quickly "walled off" or inhibited from colonisation by plant defense responses or metabolic inhibitors, and remain metabolically quiescent until the host becomes senescent; and (4) fungi that are quickly "walled off" but remain metabolically active. The major difference between these four classes of endophytes, is that fungi which extensively colonise plant tissues either avoid activation of host defense systems, or are immune to them. Endophytes having restricted host colonisation patterns, may either be activating host defense mechanisms, and in the process suppressed by these systems,

or they may be localised by the compartmentalisation of plant metabolites. Such endophytes function in a manner similar to that of host-compatible fungal plant pathogens. Endophytes in classes 3 and 4 function as host-incompatible pathogens that elicit plant defense reactions (Rodriguez and Redman, 1997).

In general the colonisation strategy of endophytic fungi is characterised by early establishment in the host tissue, followed by a quiescent biotrophic phase that may have a very long duration, although the degree of host colonisation may be limited (Stone et al., 1994). Finally, a saprophytic phase, coinciding with senescence or injury of the host , becomes evident. It is during this phase that extensive colonisation of host tissue and eventually sporulation of the fungus occurs (Stone et al., 1994)

Endophytic fungi would need specific enzymes in order to colonise various hosts and substrates under differing environmental conditions. Major exo-enzymes that are associated with the pathogenic processes of fungi include cellulase, hemicellulases, lipases, cutinases and pectinases (Griffin, 1994). These enzymes degrade plant cells both during penetration and the utilisation of cell products. Enzymes such as ß-glucosidase have the ability to degrade plant cell walls and play an important role in facilitating infection (Joseleau and Ruel, 1994)). When compared to enzymes produced by host-compatible pathogenic fungi, those of endophytes do obviously not elicit defense responses in the host plant (Rodriguez and Redman, 1997).

This study investigated the enzymatic profiles of 21 isolates of 16 species from prominent endophytic fungi isolated from *Pinus patula* Schlechtend. & Cham., *P. elliottii* Engelm. and *P. radiata* D. Don. Various saprophytic fungi, isolated from wood (2 species), and plant pathogenic fungi, isolated from other sources (2 species, one form grapes and one from groundnuts) and pine branches (1 species), and known to produce specific enzymes, were included for control purposes.

MATERIALS AND METHODS

Endophytic fungi were originally isolated from needle samples of *P. radiata*, *P. patula* and *P. elliottii* (See Chapters 2 and 3). Representative isolates of common endophytes colonising the pine needles from our previous studies, were selected for this study. Reference isolates from other sources were included for control purposes (Table 1). All isolates were tested *in vitro* for the production of the following enzymes: cellulase, pectinase, lipase, phenol oxidase, protease, ß-glucosidase, laccase, cytochrome oxidase, and peroxidase. Cytochrome oxidase, peroxidase, and laccase were tested by using "spot tests" as described by Stalpers (1978). Phenol oxidase production was tested by using the Bavendamm test as described by Rigling et al. (1989). This test together with that for lipase (Lima et al., 1991), protease and ß-glucosidase (Paterson and Bridge, 1994) were performed using agar media (7-10 ml) in test tubes (16 mm Ø) instead of Petri plates. Tests for pectinase were originally described by Hankin et al. (1971), and also previously used for studies on endophytic fungi, by Carroll and Petrini (1983). Cellulase production was tested using media originally developed by Coutts and Smith (1976), but subsequently simplified by Paterson and Bridge (1994).

Plates and tubes were inoculated with a single agar plug (5 mm Ø) colonised by a particular fungus, and incubated in the dark at 25°C for all enzymes except for pectinase which took place at 21°C. Each enzyme bioassay was replicated three times. Tubes were inspected at regular intervals and examined for a colour reaction (discoloration or clearing of the medium). Pectinase plates were flooded after 3-10 days (depending on the growth rate of the fungus) with a 1% aqueous solution of hexadecyltrimethyl-ammoniumbromide, and left for at least 2 hrs before they were examined for clearing zones around fungal colonies.

RESULTS AND DISCUSSION

Ecophysiologically, endophytes display biochemical attributes associated with needle penetration and long term residence within needles. Overall, of the 21 endophytic cultures tested, 16 produced pectinase, 21 lipase, 17 protease, 18 ß-glucosidase, 20 cytochrome oxydase and 15 peroxidase. The least conspicuous enzymes produced were cellulase (6 species), phenol oxidase (8 species) and laccase (7 species) (Table 1). *Aspergillus niger*, *Pycnoporus sanguineus* and *Gloeophyllum sepiarium* were included as positive controls for tests on cellulase production. *Pycnoporus sanguineus* was also included as a positive control for the production of phenol oxidase and laccase, whereas *G. sepiarium* was included as a negative control. The results were consistent with expected results for all the control fungi.

Endophytes living in pine needle petioles have been found to utilise a range of substrates, which include cellulose, pectin, xylan and other hemicelluloses (Carroll and Petrini, 1983). Cellulase and lipase enables endophytes to enter plants (Sieber, 1989), and are produced by both pathogenic and saprophytic fungi (Griffin, 1994). Considering that a number of endophytic fungi examined tested positive for cellulase production, it suggests that they also have at least limited saprophytic and pathogenic abilities. Two scenarios as to the role of these specific endophytes are possible: they may either be unspecialised endophytes with a limited occurrence in needles (Petrini, 1986), or they are confined to the fascicle sheath, which consists of a large proportion of cellulose (Carroll and Petrini, 1983). Most other fungi were from needle blades, which have been found to be non-cellulolytic and unable to utilise other hemicelluloses, but utilise pectin instead (Carroll and Petrini, 1983). Eighteen endophytic isolates produced ß-glucosidase. This enzyme also plays an important role in the degradation polysaccharides and specific, the cellulose components of plant cell walls, thus facilitating the infection and degradation process (Joseleau and Ruel, 1994).

Our research shows the sterile white yeast-like fungus to occur commonly in the needle

blade, but to be almost entirely absent from the fascicle sheath (See chapter 3). This is consistent with the positive pectinase production displayed by this fungus. Pectinase is also an important enzyme in the initial stages of degradation of fresh plant litter (Millar, 1974). The sterile white yeast-like fungus and other resident endophytes could thus play an important role as first colonisers of fallen needles, and posses an advantage over soil inhabiting decomposers. The production of laccase by certain fungi demonstrates their ability to degrade lignin (Griffin, 1994). This would also be an important attribute during the degradation process of senescent needles (Millar, 1974).

Lipase was produced by all isolates tested in this study (Table 1). According to Carroll and Petrini (1983) the presence of lipase suggests the ability of fungi to lyse cuticular waxes in order to have the ability to infect plant organs. Lipase, like pectinase is also an important enzyme in the infection and penetration process of plants (Griffin, 1994). Endophytic fungi would therefore be expected to produce these enzymes.

Up to four separate fungal colonies developed from a single needle section of ca. 10 mm (Kriel, Swart and Crous, unpublished results). The most prominent fungi in these sections of the needle blades proved to be *Cyclaneusma minus* and the sterile white yeast-like fungus (chapters 2 and 3). Carroll and Petrini (1983) claimed that biochemical partitioning of nutrients enabled different fungal species to colonise the same needle. Fungi that inhabit the same microhabitat usually show the same enzyme characteristics (Sieber, 1989). *Cyclaneusma minus* causes autumn needle cast, and can thus be considered a latent pathogen while the sterile white yeast-like fungus is probably a true endophyte. One would therefore expect these fungi to have different enzymatic abilities that would allow for their inhabitancy of the same needle in close proximity. The positive reaction of a number of endophytic fungi to the phenol oxydase test (Bavendamm's test) suggests their tolerance of phenolic compounds in the needles of coniferous foliage (Carroll and Petrini, 1983). The production of phenol oxydase by *Cyclaneusma minus* is especially important, since

the fungus would need to tolerate phenols present in pine needles for it's asymptomatic residence of up to 15-18 months (Kistler and Merrill, 1978). The sterile white yeast-like fungus produces pectinase, which would enable this fungus to inhabit the needle with only limited colonisation, extending at needle senescence.

Most of our results are consistent with previous studies on the same species of fungi (Carrol and Petrini, 1983; Sieber, 1989). Notably inconsistent was the negative production of cellulase by *Chaetomium* sp., which is a known cellulolytic fungus (Lakshmikant et al., 1990). A possible explanation is that the cellulose azure used our the test, consists of crystalline cellulose, which is a form of cellulose not as degradable as the amorphous fibre (Pigman and Horton, 1980). Slight differences also occurred between other isolates of the same species (see "Sterile white yeast-like fungus" in Table 1). The lack of pectinase in the endophytic isolate of *Sphaeropsis sapinea* (CEW 240), compared to the pathogenic isolate (CWS 1) may pose certain implications regarding the pathogenicity of the fungus.

Our results indicate that although endophytic fungi cause symptomless infections (except for *C. minus*), they do possess numerous exo-cellular enzymes linked to pathogenicity and limited colonisation of the needle tissue. Suppression of significant enzyme production in tissue must therefore be attributed to fungi that are either "walled off" quickly, or inhibited from colonisation by plant defense responses or metabolic inhibitors, and remain metabolically quiescent until the host becomes senescent; or fungi that are quickly "walled off" but remain metabolically active. According to Stone et al. (1994) active host reponses are triggered by initial invasion, which is possibly responsible for the restriction of endophytic colonisation. Very little evidence however, exist for such a response. This preliminary study warrants further investigation of enzymes produced by endophytes utilising more sophisticated techniques.

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Table 1. Enzymatic production by fungal isolates.

Fungus	Origin	Cellulase	Pectinase	Lipase	Phenol oxidase	Laccase	Protease	ß-glucosidase	Cytochrome oxidase	Peroxidase
Sterile black and white fungus	CEW 192	—	+	+	—	—	+	+	+	+
Alternaria tenuissima (Kunze: Fr) Wiltshire	CEW 167	—	+	+	+	+	+	+	+	+
Aspergillus niger Tiegh. *	CCP 375	+	+	+	—	—	+	—	—	—
Aureobasidium pullulans (de Bary) G. Arnaud	CEW 190	—	+	+	+	+	+	+	+	+
Botrytis cinerea Pers.: Fr. *	CCP 396	—	—	+	+	+	+	+	+	+
Chaetomium Kunze: Fr. sp.	CEW 289	—	—	+	—	+	+	+	+	+
Cladosporium Link: Fr. sp.	CEW 140	—	+	+	+	—	+	+	+	—
Cyclaneusma minus (Butin) DiCosmo, Peredo & Minter	C5t2T	—	+	+	+	—	+	+	+	—
Cyclaneusma minus (Butin) DiCosmo, Peredo & Minter	C8t2T	—	—	+	+	—	+	+	+	—
Epicoccum purpurascens Ehrenb.	CEW 266	+	+	+	—	—	+	+	+	—
Fusarium subglutinans (Wollenweb. & Reinking) P.E. Nelson, T.A. Toussoun & Marasas	CEW 150	+	+	+	—	—	+	+	+	+
Gloeophyllum sepiarium (Wulfen: Fr.) P. Karst. *	SCC 49	+	+	+	—	—	+	+	+	—
Gremmeniella M. Morelet sp.	CEW 285	+	+	+	+	+	+	+	+	+
Leptostroma Fr.: Fr. sp.	CEW 171	—	—	+	—	—	+	+	+	+
Lophodermium Chev. sp.	CEW 207	—	+	+	+	—	—	+	—	—
Nigrospora oryzae (Berk. & Boome) Petch	CEW 243	+	—	+	—	+	+	+	+	+
Nodulisporium G. Preuss. sp.	CEW 202	±	+	+	—	—	+	+	+	+
Pycnoporus sanguineus (L.: Fr.) Murrill *	SCC 87	+	+	+	+	+	+	—	+	+
Sphaeropsis sapinea (Fr.: Fr.) Dyko & Sutton. [ex. endophyte]	CEW 240	—	—	+	+	—	+	+	+	—
Sphaeropsis sapinea (Fr.: Fr.) Dyko & Sutton. [ex. *Pinus*] *	CWS 1	—	+	+	+	—	+	+	+	—
Sporormiella Ellis & Everh. sp.	CEW 222	+	+	+	—	+	—	+	+	+
Sporormiella Ellis & Everh. sp.	CEW 245	—	+	+	—	+	—	+	+	+
Sterile white yeast-like fungus	JIICa 1/7	±	+	+	—	—	—	—	+	+
Sterile white yeast-like fungus	MIDa 2/9	±	+	+	—	—	+	—	+	+
Sterile white yeast-like fungus	CEW 115	±	+	+	—	—	+	+	+	+
Sterile white yeast-like fungus	MIAb 2/12	±	+	+	—	—	+	—	+	+

* Reference isolates

SUMMARY

Endophytes, in the strict sense, are organisms that cause symptomless infections in plants. As symptomless mutualists, they can act as biocontrol agents of herbivorous insects and plant diseases. They can also be indicative of host vitality and environmental pollution. Some endophytes, however, are latent pathogens with an endophytic phase.

Pine needles are colonised by various species of endophytic fungi. It is well-known that the endophytic biota vary with host species, as well as with time. Therefore the aim of this study was to qualitatively and quantitatively compare endophyte populations within the canopies of *Pinus patula*, *P. radiata* and *P. elliottii*, during winter and summer, and within the canopies of two separate *P. radiata* trees, over different seasons. Endophytic fungi were isolated from pine needles, sampled in different seasons from various positions within the canopy, by plating surface-sterilised needle sections onto cornmeal agar supplemented with antibiotics.

In the first study a significant difference ($P < 0.05$) in endophyte numbers between samples collected during winter and summer was observed. *Pinus patula* and *P. elliottii* were more intensively colonised during winter than summer. In *P. radiata*, however, the number of fungi isolated in summer was significantly higher than in winter, but the fungal species isolated were consistent. *Cyclaneusma minus* and a sterile white yeast-like fungus were most commonly isolated. *Cyclaneusma minus* is a latent pathogen causing autumn needle cast, and sterile yeast-like fungus is suspected to be a true endophyte. Similar endophytic fungal species were isolated in the second study, performed on two eight-year-old *P. radiata* trees. Samples were taken during four seasons from an isolated, solitary tree and one growing in an even-aged, plantation nearby. Five needle fascicles of four different age groups were collected from each tree. One needle per fascicle, including the fascicle sheath, was cut into 12 sections and used for the isolations. In general, fewer endophytes were isolated from the solitary tree than the plantation tree. Qualitative and

quantitative differences in endophyte populations were observed within needles as well as between needle age groups and seasons.

The aim of the third study was to conduct a qualitative assay of enzyme production of 21 predominant fungal endophytes isolated from the pine needles. The enzymes assayed included cellulase, pectinase, lipase, laccase, phenol oxidase, protease, ß-glucosidase, cytochrome oxidase, and peroxidase. Results were consistent with attributes associated with leaf penetration and long-term residence of fungi within pine needles. All fungi screened produced at least two of the enzymes assayed. Different substrate utilisation patterns suggest biochemical partitioning of nutritional resources by endophytes. The ubiquitous presence of lipolytic activity in all isolates tested, suggests the ability to lyse cuticular waxes in order for penetration to occur. The tolerance of most tested fungi to tannic acid (phenol oxydase production) suggests low sensitivity to phenolic compounds (tannins etc.) normally present in pine needle tissue.

Management practices in plantations that reduce or increase the environmental stress on individual trees, thereby influencing endophytic populations, could have distinct beneficial or negative effects on the general vitality of trees. The most important effects of certain practices would be the influence they have on latent pathogens such as *C. minus*. Further investigation of foliar endophytic fungi of intensively managed pine plantations is therefore justified with a view to understand the effects management practices have on their ecology.

SAMEVATTING

Endofiete, in die streng sin van die woord, is organismes wat simptoomlose infeksies in plante veroorsaak. As simptoomlose mutualiste, kan hulle dien as biologiese beheeragente van plantsiektes en plantvretende insekte. Hulle kan ook 'n aanduiding gee van gasheer lewenskragtigheid en omgewingsbesoedeling. Sommige endofiete is egter latente patogene met 'n endofitiese fase.

Dennenaalde word deur verskeie endofitiese swamspesies gekoloniseer. Dit is wel-bekend dat endofitiese biota kan varieër binne gasheerspesie en ook oor tyd. Die doel van hierdie studie was 'n kwalitatiewe en kwantitatiewe vergelyking te maak van endofietpopulasies binne die blaredak van *Pinus patula*, *P. radiata* en *P. elliottii*, gedurende die winter en somer, en binne die blaredak van twee aparte *P. radiata* bome, oor verskeie seisoene. Isolasie van endofitiese swamme uit dennenaalde in verskillende seisoene en posisies binne die blaredak, is gedoen deur stukkies oppervlakgesteriliseerde naalde op mieliemeelagar met atibiotika uit te plaat.

In die eerste studie is 'n betekenisvolle verskil ($P<0.05$) in endofietgetalle waargeneem tussen monsters wat onderskeidelik in die winter en somer versamel is. *Pinus patula* en *P. elliottii* was meer intensief gekoloniseer in die winter as in die somer. In *P. radiata*, egter, was die hoeveelheid swamme in die somer geïsoleer, betekenisvol meer as in die winter, maar die swamspesie samestelling het konstant gebly. *Cyclaneusma minus* en 'n steriele wit gisagtige swam is meer gereeld geïsoleer. *Cyclaneusma minus* is 'n latente patogeen wat herfs-naaldval veroorsaak, en die steriele gisagtige swam is vermoedelik 'n egte endofiet. Soortgelyke endofitiese spesies is in die tweede studie geïsoleer. Hierdie studie is op twee agt-jaar-oue *P. radiata* bome gedoen. Monsters is gedurende vier seisoene versamel van onderskeidelik 'n alleenstaande boom, en 'n boom in 'n nabygeleë plantasie van dieselfde ouderdom. Vyf naaldbondels, van vier verskillende ouderdomsgroepe per tak, op vier takke, is uit elke boom versamel. Een naald per bondel, insluitend die bondelskede, is in 12 stukkies gesny en in die isolasies gebruik. In die algemeen is minder endofiete uit die alleenstaande boom as uit die plantasie-boom geïsoleer. Kwalitatiewe en kwantitatiewe verskille in die endofiet populasies is waargeneem, binne die naalde sowel as binne naald-ouderdomsgroepe en seisoene.

Die doel van die derde studie was om ensiem produksie van 21 mees prominente swamendofiete wat uit dennenaalde geïsoleer is kwalitatief te toets. Sellulase, pektinase, lipase,

lakkase, fenoloksidase, protease, ß-glukosidase, sitochroomoksidase en peroksidase is getoets. Die resultate was in ooreenstemming met eienskappe geassosieër met blaarpenetrasie en langtermyn inwoning van swamme binne dennenaalde. Alle swamme wat getoets is, het ten minste twee ensieme geproduseer. Verskille in substraat afbraakpatrone suggereer biochemiese onderskeiding van voedingsbronne by endofiete. Die alomteenwoordige voorkoms van lipolitiese aktiwiteit in al die bestudeerde isolate, wil te kenne gee dat hulle oor die vermoë beskik om kutikulêre was af te breek sodat penetrasie kan plaasvind. Die toleransie van meeste (van die getoetsde) swamme teenoor tanniensuur (fenoloksidase produksie), is 'n aanduiding dat hulle minder sensitief is teenoor fenoliese stowwe (tanniene ens.) wat normaalweg in dennenaaldweefsel teenwoordig is.

Bestuurspraktyke in plantasies wat omgewingstremming op individuele bome verminder of vermeerder, en sodoende endofitiese populasies beïnvloed, kan definitiewe voordelige of nadelige effekte op die algemene lewenskragtigheid van bome hê. In hierdie verband sal die invloed van sodanige praktyke op latente patogene *C. minus* van ekonomiese belang wees. Verdere ondersoeke van naaldendofiete in intensief bestuurde plantasies is daarom geregverdig met die vooruitsig om die effekte wat bestuurspraktyke op hul ekologie het, te verstaan.